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The Dynamics of Natural Enemy Resistance in Aphid Populations

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The Dynamics of Natural Enemy Resistance in Aphid Populations

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To my sons, Jake and Tom,
and to my parents, Liz and John.

“I’ve started so I’ll finish…”

Magnus Magnusson

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List of Abbreviations and Other Units

% v/v volume concentration

°C degree Celsius

kbit kilobits

μl microlitre

CO_2 carbon dioxide

Mg^{2+} magnesium

N_2 nitrogen

6-FAM 6-Carboxyfluorescein

ANOVA Analysis of Variance

APSE *Acyrthosiphon pisum* secondary endosymbiont

ATCG adenine, cytosine, guanine, thymine

BLASTN nucleotide-nucleotide Basic Local Alignment Search Tool

BLOSUM BLOcks SUbstitution Matrix

bp base pairs

CCD charge-coupled device

cm centimetre

d day

DNA deoxyribonucleic acid

dNTP	Deoxyribonucleotide triphosphate
g	gram
h	hour
IGS	intergenic spacer
JHI	The James Hutton Institute
kbp	kilo base pairs
L:D	light:dark
Mbp	mega base pairs
min	minute
ml	millilitres
mm	millimetre
MRes	Master of Research
nm	nanometre
PAXS	Pea Aphid X-type Symbiont
PCR	Polymerase chain reaction
ROX	Carboxy-X-rhodamine
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
s	second
STR	short tandem repeats
TBE	Tris/Borate/EDTA (ethylenediaminetetraacetic acid) buffer
UK	United Kingdom
UV	Ultraviolet

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Declaration

I hereby declare that the following thesis is based on the results of investigation conducted by myself, and that this thesis is my own composition. This thesis has not in whole or in any part been previously presented for a higher degree. Work other than my own is clearly stated in the text with reference to the relevant researchers or their publications. Unless otherwise stated, I have consulted all references cited.

Rebecca M. Cornwell

The relevant Ordinance and Regulations have been fulfilled by the candidate.

Supervisors

Abstract

Many aphids harbour facultative bacterial endosymbionts that can influence aphid fitness. In *Acyrthosiphon pisum* (the pea aphid), the endosymbiont *Hamiltonella defensa* confers resistance to parasitism by parasitoid wasps due to the presence of the APSE bacteriophage in the *Hamiltonella* genome. Furthermore, pea aphids that harbour both *H. defensa* and *PAXS* endosymbionts are highly resistant to the parasitoid *Aphidius ervi*. Changes in the frequency of endosymbiont infection could compromise control of aphid populations by natural enemies with implications for bio-control. Sixteen pea aphid lines were genotyped and characterised for the presence of known facultative endosymbionts and the APSE bacteriophage and, of these lines, fourteen harboured at least one known secondary endosymbiont. Intriguingly, a pea aphid clonal line harbouring a single stable infection of *PAXS* was identified. Although pea aphids harbouring a single infection of PAXS have been previously noted in the published literature, this study is the first to explore the susceptibility of such aphids to parasitoid wasps.

Experimental evidence suggests that the benefits of endosymbiont-conferred protection against parasitism come at a cost to aphid fitness. A mathematical model of the population dynamics of *A. ervi* parasitoids and endosymbiont-infected and endosymbiont-uninfected pea aphids based on published literature was further developed and refined to investigate the balance between the costs of infection and the beneficial strength of the protection and additionally to study the implications of this trade-off for endosymbiont infection frequencies in pea aphid populations. Host and parasitoid population dynamics were explored and stability boundaries between stable and oscillating populations identified. The degree of suppression of host populations by parasitoids was determined. Contrary to conclusions reported in the published literature, endosymbiont-infected and endosymbiont-uninfected aphid hosts were shown to coexist in a stable manner over a range of biologically realistic parameter values.

Further simulations were carried out using the mathematical model to explore

the effects of parasitoid choice and a strength of protection that could be overcome by superparasitism. Preliminary results suggest that this increases the potential for stable host coexistence. Using findings from the molecular characterisation of the pea aphid lines, future refinements to the mathematical model are proposed based on the potential for single protective endosymbiont infections, dual protective infections and genotypic variation in the strength of protection.

1 Introduction

1.1 General introduction

This thesis draws together experimental and theoretical work investigating the dynamics of the *Acyrthosiphon pisum-Aphidius ervi* host-parasitoid system with particular reference to the protective secondary endosymbiont bacterial infections in the pea aphid hosts.

Chapter 1 gives a general introduction to pea aphid natural enemies and the biology of the *A. pisum-A. ervi* system. The role of secondary endosymbiotic bacteria in mediating protection against parasitism by *A. ervi* wasps and the costs and benefits of infection are considered. As crop damage by pea aphids is a serious problem, the importance of natural enemy resistance within the context of biocontrol is outlined. There is a considerable history of mathematical modelling of host-parasitoid systems. The use of discrete-time models of host-parasitoid systems with an appropriate parasitoid functional response is introduced.

Chapter 2 presents the biological study system then details the molecular characterisation of the pea aphid lines held at The James Hutton Institute (JHI). After explaining an intended analysis of previously gathered data pertaining to the survival times of pea aphids of different known endosymbiont status, the methodology for preliminary assays to compare the susceptibility of pea aphid lines of different endosymbiont infection status to parasitism by *A. ervi* wasps is given. A method of rearing pea aphids in petri-dishes is presented and experimental work to measure the time taken to reach adulthood and the time taken to reach reproduction using this method is explained. Chapter 3 presents the results of the experimental work; experimental findings are discussed and a summary of main experimental conclusions given.

Chapter 4 presents a discrete-time mathematical model (arising from the work of Kwiatkowski and Vorburger (2012)) of the *A. pisum-A. ervi* system including infection of the host with horizontally and vertically transmissible protective en-

dosymbiotic bacteria. The development of the model is explained and an attempt is made to place the model within a bio-control context to assess the cost-benefits of endosymbiont in the pea aphid hosts on overall host-parasitoid dynamics. The necessary computational tools for implementing the model and interpreting the results are given.

Chapter 5 investigates variation of factors affecting the host-parasioid dynamics (informed by experimental findings) and presents results of numerical simulations. Modelling results are interpreted and discussed.

Chapter 6 brings together the experimental and theoretical work. The direction of future work is discussed, further modifications to the model suggested and overall conclusions given.

1.2 Natural enemies

The biological control of pest populations by living organisms, so called "natural enemies", uses the action of parasites (including parasitoids), pathogens and predators upon their hosts to reduce the size of pest populations and limit the damage inflicted by pest outbreaks on host plants. The natural enemies of pea aphids (figure 1.1) include parasitoid wasps, in particular the specialist parasitoid *A. ervi*, entomopathogenic fungal pathogens and generalist predators from a guild of species including *Coccinellid* and *Carabid* beetles, *Nabis* and *Orius* bugs and various spiders (Snyder and Ives, 2003).

Preservation of natural enemy communities is important as successful biological control is aided by the action of various types and species of natural enemies on pest populations, especially as a lower proportion of pests than natural enemies are typically killed by the application of broad-spectrum pesticides and pesticide residue can continue to inhibit natural enemy reproduction and parasitoid response to chemical cues vital for host location (University of California Agriculture and Natural Resources, 2007). A better understanding of host resistance to natural enemy attack is important in order to reduce or even eliminate reliance on chemical pest control.

(a)

(b)

(c)

Figure 1.1: (a) ladybird from the *Coccinellidae* family (image ©Leanne Hunter), (b) hoverfly from the *Syrphidae* family (image: JHI image archive), (c) pea aphid with fungal infection (image: David Riley, JHI).

1.2.1 Host-parasitoid systems

Predator-prey interactions form a coupled system with the dynamics of each predator and prey system relying on the outcome for the other system. Predator and prey dynamics consist of a both functional response and a numerical response. The numerical response governs the relationship between the number of predators and the density of the prey, whereas the functional response addresses the relationship between the number of prey consumed per predator and the density of the prey.

From a functional standpoint, Begon et al. (2006) identified four key types of predators within ecosystems: true predators, parasitoids, grazers and parasites. Predator interactions with already dead organisms are not considered here as prey is alive when first encountered by a predator in predator-prey or host-parasitoid interactions. Although potentially detrimental, the outcome of encounters with

grazers or parasites is hardly ever immediate prey death due to parasitism alone. After attack by a true predator, prey are usually swiftly killed and then either completely or partially consumed. Parasitoid insects lay their eggs on or in their arthropod prey. These arthropod hosts are typically parasitised at specific stages in their life cycle, most commonly at a juvenile stage (Godfray, 1998). Developing parasitoid larvae consume the host nearly entirely before undergoing metamorphosis and emerging as adult parasitoids. A true predator attack results in the death of the prey but the time taken for death to occur varies between predator types. The distinction between predator-prey interactions is not always clear in practice. Hence, the specific ecological context of the interaction must be considered.

According to Godfray (1998), about 8.5% of described insect species are parasitoids. After parasitism by an idiobiont parasitoid, hosts do not develop any further. However, parasitism by a koinobiont parasitoid permits normal, or near normal, host development for a length of time after successful attack. Koinobiont parasitoids may be endoparasitoids or ectoparasitoids, so developing in or on their hosts respectively. Pseudoparasitism is a term that is sometimes used to describe parasitoid attacks that do not result in parasitism. Without host dissection to verify that oviposition has taken place, attacks are often assumed to correspond to the oviposition of parasitoid eggs (Ives et al., 1999). Superparasitism occurs when a host is parasitised more than once. No distinction is made between self-superparasitism of the host by the same parasitoid and superparasitism by two (or more) individual parasitoids. Superparasitised hosts contain more than one lot of the usual parasitoid egg complement corresponding to each successful parasitoid oviposition. Parasitoids may also be classified as solitary or gregarious with the former resulting in the development and emergence of a single next generation parasitoid from each host and the latter giving rise to multiple next generation parasitoids from one host.

1.3 Pea aphids and parasitoid wasps: a multi-trophic system

There are approximately 4300 described species of aphids (Dixon, 1998). In arable ecosystems, aphids have key roles as vectors for disease, as prey or hosts for

predators and as herbivores. Aphids are hemimetabolous. With no pupal stage, these soft-bodies insects pass through incomplete metamorphosis from the juvenile to adult stage. The pea aphid (*Acyrthosiphon pisum*; Hemiptera: Aphididae) is amongst the bigger aphid species. They are a model organism in laboratory studies. The pea aphid complete draft genome (464 Mbp) was sequenced by The International Aphid Genomics Consortium (2010) and is the first published whole genome sequence of a basal hemimetabolous insect. Pea aphids feed only on plant phloem sap. This feeding causes direct damage resulting in stunted host plant growth, distortion and discolouration of leaves and pods. Populations peak during late June and early July and maximum impact on crop yields will occur when the population peak coincides with crops coming into flower. Damage to crops also occurs because pea aphids can transmit in excess of 30 plant viruses with ecological and economic consequences (Rohamsted Research, 2015).

Aphids are habitually attacked by a variety of parasitoid wasps (Gwynn et al., 2005). *Aphidius ervi* (Hymenoptera: Braconidae: Aphidiinae) parasitoid wasps are koinobiont endoparasitoids (Quicke, 1997), developing within the host body, and are used for aphid biological control. In this study system, superparasitism is deposition of an *A. ervi* wasp egg into an aphid host already parasitised by an *A. ervi* wasp. *A. ervi* is a solitary parasitoid so deposits a single egg during each oviposition and only one wasp larva survives in any superparasitised pea aphid host.

Pea aphids show phenotypic plasticity (The International Aphid Genomics Consortium, 2010) and, depending on the season, pea aphids may be asexual viviparous females, egg-laying females or sexual males. Asexual females take two forms: apterous (wingless) or alate (winged). Alates can, if necessary, disperse to other host plants due to overcrowding. The pea aphid lifecycle is typically holocyclic, alternating at least 1 generation of asexual reproduction (parthenogenesis) with a single generation of sexual reproduction over winter. Parthenogenetic female aphids give birth, when reproducing asexually, to live nymphs. Akin, in an everyday context, to a matryoshka doll, these daughters are born containing the developing embryos of their daughters within their ovarioles that in turn harbour the developing embryos of *their* daughters. An holocyclic life cycle provides the pea aphids with an evolutionary advantage. Genetic recombination (resulting in increased genotypic variation during sexual reproduction), together with high reproductive output due to telescopic generations during asexual reproduction, lead

to rapid population growth triggered by suitable environmental conditions with consequences for the life traits and population dynamics of their natural enemies (Le Ralec et al., 2010).

An aphid nymph will moult its exoskeleton (ecdysis) during larval development. The definition of an instar is the period between moults or between final moult into adulthood and pea aphids have 4 larval instars. First instar nymphs are born live during asexual reproduction. The stage of larval development can be determined from aphid siphuncular (cornicle) length (Hutchinson and Hogg, 1983). Pea aphids occur as either green or pink colour morphs with adult apterae sizes ranging from 2.5 mm to 4.4 mm and alatae size ranging from 2.3 mm to 4.3 mm (Blackman, 1984). Minks and Harrewijn (1987) state that the first, second and third instars of apterous adults are of approximately constant time period under laboratory conditions. Further, they found that alatae have a prolonged fourth instar of more than 24 h longer at 20 °C compared to the fourth instar of apterae. The divergence in development times between alatae and apterae continues into the prelarviposition period. At 20 °C, apterae commence larviposition 0.5 d to 3 d after emerging as adults. However, in alatae, the prelarviposition period is more variable, as environmental conditions may force dispersal to other host plants before the start of nymph deposition.

Parasitoid wasps use alarm pheromone cornicle emissions from pea aphids to find aphid hosts (Godfray, 1998). The pheromones stimulate an intense oviposition attack reaction from *A. ervi* female wasps (Le Ralec et al., 2010). Using a specialised ovipositor, female parasitoid wasps sting aphids and inject their eggs. The sting causes temporary paralysis of the host (Godfray, 1998). Following attack and oviposition by koinobiont *A. ervi* wasps, the aphid host continues with larval development, even reaching reproduction, before the growing wasp larva devours its vital organs. The aphid host is now a husk known as a "mummy". The final stage in wasp development is pupation within the mummy. The reproductive adult wasp emerges from the aphid mummy approximately 10 d to 14 d after oviposition. However, aphid hosts may attempt to fight back when encountered by a parasitoid. Individual aphids may resist parasitoid attack (Dixon, 1998) using three primary defence mechanisms. Aphids may remove their feeding stylets from the host plant and remain rigid, or may run away from the parasitoid, or drop from the plant (Godfray, 1998).

1.3.1 Aphid endomicrobiota and bacteriophages

There are two categories of symbiotic bacteria. These are obligate primary endosymbionts and facultative secondary endosymbionts. Most aphids, including pea aphids, harbour the obligate primary endosymbiont *Buchnera aphidicola* (gamma proteobacteria). *Buchnera* is an intracellular microorganism viable only within the cytoplasm of cells called bacteriocytes or mycetocytes. It is vertically transmitted from host mother to nymph either through oogenesis in sexual reproduction or during embryogenesis in asexual reproduction. The pea aphid host and *Buchnera* symbiont cannot survive without each other so demonstrating an example of a mutualistic symbiosis (Futuyma, 1998). Pea aphids provide a stable ecological niche for *Buchnera* comprising a supply of nutrients from the aphid host in return for synthesis of essential amino acids and other nutrients by endosymbiont absent from the host diet (Clark et al., 2010).

The benefits of secondary facultative endosymbionts infection typically vary with the ecological environment that the host inhabits. Hence, the returns can vary in both space and time. Such variation has consequences for the ecological community (Ferrari and Vavre, 2011). To date, the most studied secondary facultative endosymbionts of the pea aphid, *Hamiltonella defensa*, figure 1.2[1], *Serratia symbiotica* and *Regiella insecticola*, are, like *Buchnera*, gamma proteobacteria within the *Enterobacteriaceae* family. Whilst *H. defensa* and *R. insecticola* are sister groups related to species of *Photorhabdus* bacteria, phylogenetic analysis has shown that *S. symbiotica* belongs to the free-living *Serratia* species group (Moran et al., 2005a). Other described secondary endosymbionts found in pea aphids are an alpha proteobacteria *Rickettsia* (Sakurai et al., 2005), a gamma proteobacteria *Rickettsiella* (Tsuchida et al., 2010), a *Spiroplasma* (Fukatsu et al., 2001) and another gamma proteobacteria called the pea aphid X-type symbiont (PAXS) or the *X-type* (Guay et al., 2009). Frantz et al. (2009) report that as many as three facultative endosymbionts may co-infect a pea aphid clonal line.

Viruses that infect bacteria are called bacteriophages. New genomic technologies have revealed that virus genomes are pervasive within bacterial genome sequences. As such, Casjens (2003) suggests that it is important to identify viral genes and the mechanisms by which they affect their hosts. It was van der

[1]Reprinted from Virology, 262, van der Wilk et al, Isolation and characterization of apse-1, a bacteriophage infecting the secondary endosymbion of *Acyrthosiphon pisum*, 104-133, Copyright (1999), with permission from Elsevier.

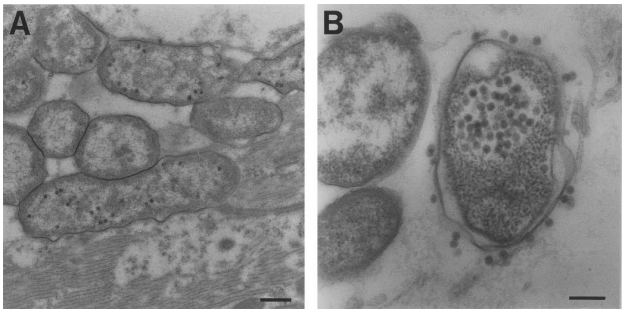


Figure 1.2: Electron micrograph of secondary endosymbiotic bacteria in pea aphids

Wilk et al. (1999) who first identified and characterised lysogenic lamdoid bacteriophage particles visibly discernible in electron micrographs of pea aphid secondary endosymbiont bacterial cells and morphologically similar to the *Podoviridae* species. This bacteriophage, figure 1.3[2], is referred to as bacteriophage 1 of the *Acyrthosiphon pisum* secondary endosymbiont (APSE). Degnan and Moran

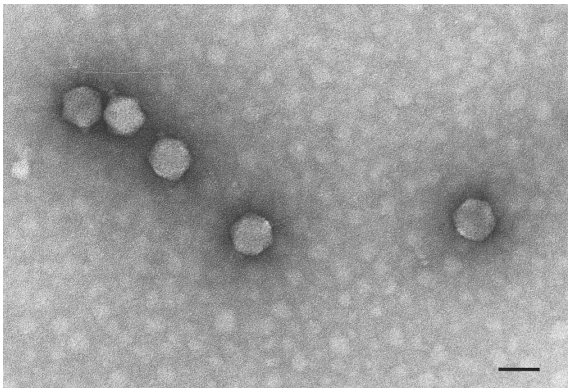


Figure 1.3: Electron micrograph of APSE bacteriophage

(2008) identified primers from 5 APSE genes distributed along the 36 kb to 40 kb APSE genome using deoxyribonucleic acid (DNA) from *A. pisum* and other aphids species. The P35 APSE gene is suggested to be associated with injecting phage DNA into host cells and was found to be highly conserved. The P51 gene associated with regulation was not highly conserved. These gene fragments have product sizes 822 bp and 813 bp respectively. In their study, APSE was found to be present in all *H. defensa* infections, contrary to the findings of Sandström et al.

[2]Reprinted from Virology, 262, van der Wilk et al, Isolation and characterization of apse-1, a bacteriophage infecting the secondary endosymbiont of *Acyrthosiphon pisum*, 104-133, Copyright (1999), with permission from Elsevier.

(2001) and Moran et al. (2005b). Hence, Degnan and Moran (2008) assert that the presence of APSE cannot be determined from diagnostic polymerase chain reaction (PCR) alone, but that intergenic amplifications or Southern hybridisations are necessary.

In a recent review paper, Vorburger (2014) reports that 3 variants of the APSE bacteriophage in *H. defensa* (APSE1, APSE2 and APSE3) have been identified so far with each encoding a distinct toxin gene thought to be fatal to the oviposited parasitoid wasp egg or larvae. Further, experimental evidence suggests that the among-strain protection conferred by *H. defensa* with APSE is dependent on the variant of APSE present. Interestingly, although similar among-strain protection has been noted in *A. fabae*, protection against parasitism does not appear guaranteed by an *H. defensa* with APSE infection, with Vorburger (2014) citing the grain aphid *Sitobion avenae* as lacking a significant protection when attacked by *A. ervi*.

1.3.2 Resistance to parasitism

Gerardo et al. (2010) recap the various defence mechanisms utilised by insects. If primary behavioural defence strategies fail to be effective, insect physiological defences are tested. The next line of defence is immunological. These innate defences are cellular (phagocytosis and encapsulation), clotting and the generation of antimicrobial substances. Inspection of the pea aphid genome suggests that genes found in other insect genomes that are purported to be crucial for defence against microbes are missing from the pea aphid defence armoury. Gerardo et al. (2010) assert that the close relationship between pea aphids and their endomicrobiota helps the aphids endure without a robust immune defensive response.

A small genome is indicative of a facultative endosymbiont role (Moran et al., 2005b). The complete genome of *Buchnera* was sequenced (Shigenobu et al., 2000) and found to be approximately $641\,\text{kbp}$. The *H. defensa* genome (approximately $1.7\,\text{Mbp}$) is much smaller than genomes of free living *Enterobacteriacae (*typically $4\,\text{Mbp}$ to $6\,\text{Mbp}$). When *H. defensa* samples were sequenced, high numbers of APSE phage genomes were found throughout the samples. Hence, the *H. defensa* genome is not as greatly reduced as that of the obligate primary endosymbiont *Buchnera* which does not contain phage genes.

Pea aphid host resistance to parasitism by *A. ervi* parasitoid wasps is considered to be due to the presence of the APSE bacteriophge in the *H. defensa* endosymbiont

(Oliver et al., 2009) within the pea aphid host (section 1.3.1). It is further hypothesised that the protection may specifically arise from the inclusion of genes homologous to toxin-encoding genes in mammalian pathogens (Degnan et al., 2009) in the APSE phage genome. In addition to resistance demonstrated in singly-infected pea aphid lines, the co-infections *H. defensa-Spiroplasma*, *R. insecticola-H. defensa* and *S. symbiotica-H. defensa* (Oliver et al., 2006) have also been reported to confer varying degrees of resistance against parasitism. In experimental assays, Guay et al. (2009) demonstrated very high resistance (100%) in pea aphid clones infected with both *H. defensa* and PAXS.

Whilst infection with *S. symbiotica* (Oliver et al., 2003), with *R. insecticola* (Vorburger et al., 2010) and with the co-infection *R. insecticola-Spiroplasma* (Oliver et al., 2003) have also been reported to confer varying degrees of resistance in *A. pisum* against parasitism by *A. ervi*, studies by Nyabuga et al. (2010) and Guay et al. (2009) found that *S. symbiotica* did not confer any statistically significant resistance to parasitism.

Findings from genomic sequencing of the pea aphid and its obligate and facultative bacterial endosymbionts have both led to a deeper understanding of the symbiotic relationship between host and endosymbionts and have also given rise to fascinating questions about complex multi-trophic level interactions between aphids, their endomicrobiota and their natural enemies. The ability to genotype aphids (and their natural enemies) opens up new avenues for investigation into host-parasitoid interactions. Rather than relying on physical characteristics such as colour morph or molecular characteristics such as endosymbiont complement to identify aphid clonal lines, genotyping using microsatellite markers allows for clear distinction between aphid lines and the identification of aphids of the same genotype with different endosymbiont infections. This allows for investigation into genotype-endosymbiont associations. With genotyping becoming established practice, interesting observations about potential genotypic rather than endosymbiont-mediated bases for parasitoid resistance are being noted. Vorburger (2014) points out that the amount of protection conferred by a strain of *H. defensa* may depend on the genotype of the attacking parasitoid in addition to any among-strain variation due to the APSE variant associated with the *H. defensa* infection.

1.3.3 Costs and benefits of endosymbiont infection

Pea aphids have been the model for studying the impact of facultative endosymbionts and APSE on aphid fitness. Some studies (Oliver et al., 2005, 2006, 2003) suggest fitness benefits may be conferred to infected parasitised aphids. However, evidence that harbouring secondary endosymbionts such as *H. defensa* may lead to fitness trade-offs in aphids (Gwynn et al., 2005) has also been presented. Research carried out for an MRes project (Cornwell, 2011) found that a particular pea aphid clonal line harbouring *H. defensa* and PAXS exhibited the benefit of total resistance to parasitism (also found by Guay et al. (2009)) but at a cost to aphid fitness. Survival analysis of clonal lines used during the study showed a significant difference between aphid clonal lines at the 1% level. The pea aphid clonal line with a dual infection of *H. defensa* with PAXS survived for significantly shorter times. It was also found that aphids infected with *H. defensa* had a significantly lower intrinsic population growth rate at the 5% level than aphids harbouring *S. symbiotica*. Lastly, a significant difference was noted between endosymbiont infection status (*H. defensa* infection or *S. symbiotica* infection or unknown/no infection) in aphid resistance to parasitism at the 10% level in a parasitism arena experiment. This evidence suggests that fitness trade-offs may exist within the *A. pisum-A. ervi* biological study system.

The coupling of population dynamics through the host-parasitoid interaction may allow aphid endosymbiont infection to shape parasitoid ecology (section 1.3). The prevalence of such infection in pea aphid populations may change aphid host and parasitoid wasp population dynamics through increased natural enemy resistance but at a cost of host fitness. Looking to the implications of this interaction in the wider ecosystem, this may affect the severity of aphid crop damage and the impact of natural biological control measures with environmental and economic consequences of interest to both agricultural and scientific communities.

1.3.4 Horizontal and vertical transmission: acquisition and loss of facultative endosymbionts

Vertical inheritance of endosymbionts occurs during reproduction. As discussed in section 1.3, reproduction is either asexual during parthenogenetic viviparous summer generations or sexual with transmission occurring via diapausing eggs

over winter. Vorburger (2014) reviewed the available information on the fidelity of vertical transmission during parthenogenetic generations and concludes that there are no reports, in laboratory conditions, of the loss of *H. defensa*, although the spontaneous loss of other endosymbionts from an initially double-infected aphid has been reported. Further, the fidelity of vertical transmission via eggs may be slightly less than perfect, but this is only a tentative conclusion and Vorburger (2014) highlights the dearth of knowledge about vertical endosymbiont gain and loss of secondary endosymbionts under field conditions and the subsequent ramifications for our understanding endosymbiont-mediated dynamics.

Possible routes for horizontal transmission of secondary endosymbionts are thought to be via sexual transfer (from males to females), transfer by parasitoids (akin to the "dirty needle" vector) and by ingestion. Sexual transfer of *H. defensa* in pea aphids (Moran and Dunbar, 2006) and transmission of *H. defensa* by ingestion by manipulation of pea aphid artificial diets (Darby and Douglas, 2003) have been reported; infection via parasitoids has not been reported in pea aphids. Vorburger (2014) suggests that other mechanisms for horizontal transmission are yet to be discovered and that there is considerable uncertainty over the rate of horizontal infection via any of these routes.

1.4 Bio-control

Snyder and Ives (2003) summarise the key characteristics of proficient natural enemies when controlling host populations. Unlike generalist predators, specialist predators have high host specificity, substantial reproductive potential and rapid generation time. *A. ervi* satisfies these effectiveness criteria: they prey upon various aphids (Quicke, 1997) with female parasitoids attacking many hosts over a lifetime, and grow from egg to emerging adult within the aphid host so correlating host and parasitoid development times. The potential for a substantial numerical response (section 1.2.1) to a sudden increase in host population size is thought to be responsible for the success of deliberately introduced parasitoids against outbreaks of agricultural crop pests.

The heterozygosity of *A. ervi* held in laboratory lines was shown to halve by a length of time in culture equivalent to about 9 months. This has implications for the rearing and supply of populations of *A. ervi* for biocontrol (Quicke, 1997).

1.5 Mathematical models of host-parasitoid interactions

1.5.1 Population growth and stability

To understand the discrete-time predator-prey models described later, it is helpful to start by considering the dynamics of host population growth in the absence of predators. Density independent population growth can be of value early in the modelling process (Alstad, 2001). Modifications can be added to a simple model to make it an increasingly closer approximation to reality. In a density-independent population model, the rate of population growth per capita is independent of population density, hence an individual's reproduction, development or survival is not affected by other individuals within the population or by any limits to required resources. Key assumptions are: a population size governed only by births and deaths (with no net change due to immigration or emigration), the probability of each individual reproducing or dying is equal across the population, reproduction is asexual (hence complexities due to mating can be ignored) and necessary environmental resources are unlimited.

The life history of organisms in a population is important, as this determines whether the population growth should be modelled as discrete or continuous. Organisms that reproduce seasonally are modelled by discrete (geometric) growth, whereas organisms that breed continuously, and hence have overlapping generations, are modelled by continuous growth. Mathematically, the difference between approaches is that discrete growth is based on a difference equation whereas continuous growth is based on a differential equation.

In contrast to the simple density-independent population model outlined earlier, the rate of population growth per capita in a density-dependent population *can* be affected by population density. The main cause of this is intra-specific competition. Hence, a density dependent feedback term is added to the model and the assumption of the density independent model of infinite resources is dropped. If population growth was continuous, the effects of density-dependency would be felt instantaneously. However, due to the nature of discrete dynamics, there is a time lag in its effects. The maximum prey population that can be sustained is called the carrying capacity of the population.

For different parameters, the stability of a model can be interpreted as a mea-

sure of the resilience of the ecological system. A system is in equilibrium when counteracting processes balance. When a system is in stable equilibrium, the populations will return to the same values if disturbed or perturbed. The return to equilibrium is either monotonic or as damped oscillations. Likewise, if a system is exhibiting stable cycles, it will return to the same behaviour if disturbed. Beyond this point, a population will display dynamics described mathematically as chaotic. When chaotic, populations oscillate in such a way that they never follow a precisely repeating pattern and systems that appear identical at the start can subsequently vary in totally different patterns simply due to the acute sensitivity of a chaotic system to variations in its initial conditions. As a consequence, the population size that a chaotic system will reach at some future time cannot be predicted. If the equilibrium is unstable, populations will not return to the same values if disturbed.

1.5.2 Host-parasitoid models

The work of Nicholson and Bailey (1935) underpins a vast body of work in this field. The collaboration of Nicholson, an Entomologist, with Bailey, a Physicist, is discussed within the context of the history of ecological modelling by Kingsland (1988). Nicholson's initial model grew from hypothetical examples which were then expressed in mathematical form by Bailey. Their model is discrete-time rather than continuous-time as the legacy of the way in which Nicholson deduced his first arguments. Despite the model not being based on experimental data, predator-prey dynamics following the density independent Nicholson-Bailey model have been observed under laboratory conditions with careful experimental design. An example of this is the experiment carried out by Burnett (1958) who studied the interaction between greenhouse whitefly *Trialeurodes vaporariorum* and its parasitoid *Encarsia formosa*. As predator-prey dynamics are difficult to study in a controlled manner in nature, computer models can be used to carry out a large number of simulations in a short time. May and Hassell (1981) explore the stability of a simple multipredator system. Later, Hassell and May (1986) present an exploration of the dynamics of specialist and generalist natural enemy interactions within the context of a host-parasitoid model. Building from work such as Hassell and May (1973), models such as those by Sherratt (2001) that exhibit oscillatory temporal dynamics are a topic of interest in the literature.

The basic Nicholson-Bailey model of predation has two key assumptions. Nicholson and Bailey (1935) assumed that the number of parasitoids is due only to random encounters with hosts and that, save for losses to the host population due these random encounters, the host population grows exponentially. If parasitoids encounter hosts at random, the Poisson distribution describes the proportion of hosts found each time. The model is expressed as follows:

$$\left.\begin{aligned} H(t+1) &= H(t)\exp\left\{r - AP(t)\right\} \ , \\ P(t+1) &= H(t)\left\{1 - \exp\left(-AP(t)\right)\right\} \ , \end{aligned}\right\} \tag{1.1}$$

where $H(t)$ is the number of hosts and $P(t)$ is the number of parasitoids in generation t, A is the parasitoid search efficiency and r is the intrinsic growth rate of the host population (Begon and Mortimer, 1986).

Brassil and Abrams (2004) extended the basic Nicholson Bailey model to a two-host one parasitoid system; Hassell (1978) considered a general one-host two parasitoid systems; Hogarth and Diamond (1984) present a discrete one host, two parasitoid system. Kwiatkowski and Vorburger (2012) propose a discrete time model of coupled host-parasitoid population difference equations with subclasses of symbiont-free and symbiont-harbouring hosts to model wasp-aphid-endosymbiont temporal population dynamics with time-lags between parasitised host populations and parasitoid populations. This model is directly relevant to the multi-trophic system studied at JHI and seeks to explore the fitness costs of harbouring endosymbionts. The model presented by Kwiatkowski and Vorburger (2012) is complicated due to the biological complexity of the system, but it yields interesting results in terms of the stability of the system and the magnitude of the parameters needed for infected and uninfected aphid hosts to co-exist. The paper relies heavily on data for *A. fabae*, but data from experimental work at JHI shows different parameters ranges for some of the parameters used in the model.

1.5.3 Parasitoid functional responses

Hassell (1978) reports three different responses for the number of prey eaten per predator as the prey density increases as described by Hollings in 1959. With a type I response (as in the basic Nicholson Bailey model described by equations 1.1), the rate of prey consumption by predators increases linearly as the prey

density increases. With a type II response, the rate of consumption increases with prey density but the rate gradually slows down until a plateau is reached. With a type III response, the rate of consumption follows a sigmoidal (S-shaped) trend as prey density increases. For both type II and type III responses, the plateau is reached when all the predators are satiated because of the abundance of prey so the proportion of available prey killed decreases and, as a result, the number of prey not attacked increases.

The modified Nicholson Bailey models incorporating type II (equations 1.2) and type III (equations 1.3) functional responses are therefore of the form (Begon and Mortimer, 1986):

$$\left.\begin{array}{rcl} H(t+1) & = & H(t)\exp\left\{r-\frac{aTP(t)}{1+aT_hH(t)}\right\}\ , \\ P(t+1) & = & H(t)\left\{1-\exp\left(\frac{-aTP(t)}{1+aT_hH(t)}\right)\right\}\ , \end{array}\right\} \tag{1.2}$$

where $H(t)$ and $P(t)$ are again the host and parasitoid population sizes in generation t, a is the instantaneous parasitoid search rate, T is the total parasitoid search time and T_h is the handling time (the time taken by a parasitoid to attack a host),

$$\left.\begin{array}{rcl} H(t+1) & = & H(t)\exp\left\{r-\frac{xTH(t)P(t)}{1+yH(t)+xT_hH(t)^2}\right\}\ , \\ P(t+1) & = & H(t)\left\{1-\exp\left(\frac{-xTH(t)P(t)}{1+yH(t)-xT_hH(t)^2}\right)\right\}\ , \end{array}\right\} \tag{1.3}$$

with $H(t)$, $P(t)$ and T_h as above and a replaced by a function of the form

$$a=\frac{xH(t)}{1+yH(t)}\ , \tag{1.4}$$

where x and y are constants.

There is some debate as to whether type III functional responses are exhibited by predators that learn. This learning behaviour was described by Hollings in relation to small mammals, however Hassell (1978) gives examples of type III functional responses exhibited by arthropods and parasitoids. Differences in type II and type III responses have been attributed to learning time and/or prey switching where a predator consumes more than one species of prey depending on availability. Type III functional responses have the potential to stabilise predator-prey interactions but not in the single predator-prey model. Zimmer (1999) reports findings from an ecology experiment examining the dynamics of a predator within a basic food

chain suggesting that the chaotic dynamics possible when predator populations depended solely on one prey species were lost when interactions with other species were introduced to the model.

The Ivlev functional response (1.5) describes the number of prey encounters per predator:

$$N_e = c_{\max}\left(1 - \exp\left(-bN\right)\right) \ , \tag{1.5}$$

where N_e is the number of prey encountered per predator, $c_{\max}$ is the maximum number of prey consumed by an individual predator per unit time and b is the steepness of the curve (Crawley, 1992). An alternative to the Hollings type II functional response generally associated with invertebrate behaviour, the Ivlev functional response has been used in models of crop pests (Gutierrez et al., 1998) although it more usually applied to situations where predator uptake of prey is limited by predator gut capacity rather than cases where handling and search time are clearly delineated (Crawley, 1992).

1.5.4 Host refuges

Total safety from predation in the natural world is rare, however partial refuges from predation much more commonly arise (Begon and Mortimer, 1986). Refuge protection may be conferred to either a constant number of hosts or a constant proportion of hosts with both factors stabilising host-parasitoid interactions (Hassell and May, 1973) with a constant number of host refuges providing the greatest stabilising effect.

Introduction of a constant proportion refuge (Begon and Mortimer, 1986) into the basic Nicholson Bailey model gives:

$$\left.\begin{aligned} H(t+1) &= (1-\gamma)H(t)\exp(r) + \gamma H(t)\exp\left\{r - AP(t)\right\} \ , \\ P(t+1) &= \gamma H(t)\left\{1 - \exp\left(-AP(t)\right)\right\} \ , \end{aligned}\right\} \tag{1.6}$$

where γ is the proportion of hosts outside the refuge and subject to parasitism.

1.5.5 Encapsulation as partial refuge from predation

Encapsulation, in pea aphids, is a cellular immunological defence response (section 1.3.2) mounted against oviposited parasitoid eggs within the aphid host. Godfray and Hassell (1991) explain that parasitoid eggs may be oviposited into a host that is too immature to provide the resources necessary for the growth of the parasitoid. In such circumstances, the parasitoids quiesce at either the egg or first larval stage of development until the host has reached a stage of development compatible with continued parasitoid growth. During encapsulation, specialist cells in the aphid haemocoel accumulate and harden around the developing parasitoid forming a capsule which asphyxiates or starves the parasitoid to death. For modelling purposes, encapsulation may be considered as either "all-or-none" with hosts overcoming parasitism by encapsulation with a constant probability regardless of the number of parasitoid larvae oviposited over time (hosts are either not encountered by parasitoids or encountered one or more times) or "dosage-dependent" where the host's risk of being killed by parasitism increases with the number of parasitoids oviposited over time (Godfray and Hassell, 1991).

If, in its most basic form, the Nicholson Bailey model is written as:

$$\left.\begin{aligned} H(t+1) &= H(t)\lambda f(H(t),P(t)) \ , \\ P(t+1) &= H(t)\left(1-f(H(t),P(t))\right) \ , \end{aligned}\right\} \tag{1.7}$$

where λ is the host net fecundity and $f(H(t),P(t))$ is the fraction of hosts escaping parasitism. Then the introduction of a constant probability of hosts overcoming parasitism by "all-or-none" encapsulation of oviposited parasitoids is:

$$\left.\begin{aligned} H(t+1) &= H(t)\lambda\left[f(H(t),P(t))+\eta\left(1-f(H(t),P(t))\right)\right] \ , \\ P(t+1) &= H(t)\left(1-\eta\right)\left(1-f(H(t),P(t))\right) \ , \end{aligned}\right\} \tag{1.8}$$

where η is the proportion of hosts protected from parasitism.

Comparison of expressions 1.6 and 1.8 shows clearly that protection from parasitism by the mechanism of encapsulation is analogous with the safety of a partial physical refuge from predation.

Godfray and Hassell (1991) show that, in the case of "all-or-none" encapsulation, fluctuation in the effectiveness of the encapsulation response does not affect the dynamics of this simple host-parasitoid system. Further, in the case of "dosage-

dependent" encapsulation, the parasitoid search efficiency, A (see equation 1.1), is reduced by $(1 - \eta)$ leading to higher host and parasitoid equilibrium populations, but not affecting the stability of the system. If fluctuation in the effectiveness of the encapsulation response is introduced to the "dosage-dependent" model, the resulting dynamics are more complex.

Godfray and Hassell (1991) then consider encapsulation within an evolutionary context and postulate a trade-off between the benefits of an encapsulation response and unspecified fitness costs to the host. Their hypothesis pre-dates the bulk of the work on secondary endosymbionts in pea aphids and associated costs and benefits of harbouring such infections. However, the extension of their postulate to include costs and benefits of endosymbiont mediated protection is a logical progression that can now be considered. For modelling purposes, resistance to parasitism by encapsulation whether innate or enhanced by infection is still analogous to a partial refuge from predation. Godfray and Hassell (1991) describe the host encapsulation response in terms of η which is a measure of the strength of either the "all-or-none" or "dosage-dependent" response, and λ which is the host net fecundity. The authors make the broad assumption that, as host fecundity increases, the encapsulation ability of the host decreases. The authors go on to consider the fate of a "resident clone" host, N, when invaded by a "mutant clone" host, S of differing encapsulation ability and fecundity (η_1, λ_1 and η_2, λ_2 respectively). This is a similar question to that posed by Kwiatkowski and Vorburger (2012) who consider the fate of uninfected and infected host populations with varying strength of protection against parasitism (akin to η_1 and η_2) and constitutive cost incurred of harbouring a secondary endosymbiont infection (affecting fecundities, λ_1 and λ_2). Following numerical simulations, Godfray and Hassell (1991) report that, in the simple case of an evolutionary strategy solely determined by the host-parasitoid dynamics of equation 1.8, there is no evidence for the persistence of polymorphism in encapsulation ability in host populations and, dependent on the trade-offs between encapsulation ability and fecundity, either the "resident" or "mutant" clonal host populations survive. It is possible for the "mutant" hosts to displace the "resident" hosts; this outcome is possible with final populations dynamics of either a stable equilibrium or limit cycles.

A further modelling complexity is then introduced by Godfray and Hassell (1991)

in the form of density dependence in the host populations of the form:

$$g\left(N_t, S_t\right) = \exp\left(\frac{-\left(N_t + S_t\right)}{K}\right) , \tag{1.9}$$

where K is the carrying capacity of the host populations. The equations from the Godfray and Hassell (1991) study of relevance here are those that describe the population dynamics of the resident and mutant clonal host populations and the parasitoid population:

$$\left.\begin{aligned} N_{t+1} &= N_t \lambda_1 g\left(N_t, S_t\right)\left[f(P_t) + \eta_1\left(1 - f(P_t)\right)\right] , \\ S_{t+1} &= S_t \lambda_2 g\left(N_t, S_t\right)\left[f(P_t) + \eta_2\left(1 - f(P_t)\right)\right] , \\ P_{t+1} &= N_t\left(1 - \eta_1\right)\left[1 - f(P_t)\right] + S_t\left(1 - \eta_2\right)\left[1 - f(P_t)\right] . \end{aligned}\right\} \tag{1.10}$$

Godfray and Hassell (1991) found similarities between this model and their simpler density independent model in that "mutant" hosts with either a marginally higher fecundity or encapsulation ability take over the "resident" host populations. However, they express surprise on finding coexistence between "resident" and "mutant" populations when "mutant" clones have a much higher encapsulation ability and lesser fecundity. Coexistence was exhibited by both stable and oscillating host populations. The authors acknowledge that further work in needed to explore the mechanism by which density dependence apparently introduces polymorphism of encapsulation ability as an evolutionary outcome.

It would seem that Kwiatkowski and Vorburger (2012) did not base their work directly upon that carried out by Godfray and Hassell (1991) as they do not cite this work but this study brings together the two modelling approaches. Kwiatkowski and Vorburger (2012) concluded that stable coexistence between uninfected and infected host populations was not a possible outcome; coexistence was only observed in oscillating host populations and infected hosts always completely displaced uninfected hosts when in stable equilibrium. This contradicts the findings of Godfray and Hassell (1991). Given the failure of pea aphid hosts with beneficial endosymbiont-conferred resistance to parasitism to displace uninfected hosts in natural populations, this discrepancy is worthy of further investigation.

1.5.6 Modelling approaches

Levins (1966) tackles the competing priorities of population modelling. Although his analysis of the pros and cons of different approaches to complex modelling was carried out at an earlier stage in the development of this field, the issues are still highly relevant today and, if anything, magnified by the computational ability of modern computers and software. The broad modelling approaches scrutinised are the "naive, brute force" approach consisting of a highly complex but faithful mathematical reproduction of a biological system, or simplified approaches forgoing generality in favour of realism and precision, or forgoing realism in favour of generality and precision or, finally, forgoing precision to realism and generality. Levins (1966) calls for the identification of robust ecological theorems using different modelling strategies (with associated varying simplifications) to test the same underlying biological assumptions. When a robust theorem is modelled, the results are not crucially dependent on the details of the mathematical model employed. The models discussed in this study broadly fall into the third category described by Levins (1966) namely "sacrifice realism to generality and precision". Simulations of such models based on general mathematical equations appropriately parameterised give highly similar results when conditions are unchanged allowing for quantitative predictions. Comparison of simulation results with field data may show where theory and reality diverge and this may inform modifications to the mathematical model. The aim of this type of model is to move *towards* modelling with increasing realism.

The Nicholson Bailey model and subsequent modifications are based on host parasitoid systems with non-overlapping generations and are often applied to scenarios where there is one generation per time interval. Cobbold et al. (2009) confirm the suitability of the application of discrete-time models to insect systems with non-overlapping generations in temperate environments. As discussed in section 1.3, the *Acyrthosiphon pisum-Aphidius ervi* host-parasitoid system in the UK consists of at least one generation of asexual reproduction punctuated by a sexual generation over winter. In the field, it is possible for the host parasitoid system to start with non-overlapping generations but to lose this synchronicity through time. During winter, the parasitoids enter diapause. Pea aphids held in culture typically rely on asexual reproduction throughout the year to maintain the integrity of the clonal lineage and parasitoids are typically reared in separate culture with host

and parasitoid organisms of a pre-selected age introduced to each other for the first time at the start of experimental work. Whilst experimental variables such as temperature, light, plant type and quality can be closely controlled in the laboratory, field conditions are diverse and more challenging for both experimental work and modelling. The models so far discussed in this study are deterministic in nature rather than stochastic with the latter falling outwith the scope of this project. The models considered here are also temporal rather than spatio-temporal. Extending the analogies considered in sections 1.5.4 and 1.5.5, partial refuges from predation arising through the protection conferred by secondary endosymbiont infection may be considered equivalent in effect to both physical spatial refuges from predation and to partial refuges due to encapsulation.

In addition to excluding environmental variability, the emphasis in the host-parasitoid modelling process so far described has been on the factors affecting population growth of the host and on the functional response of the parasitoid predator which in turn relates to the parasitoid population growth rate. This simplistic approach to parasitoid population modelling can be of benefit depending on the focus of the modelling process. However, the relationship between host death and rate of parasitoid population increase is also dependent on factors including the number of parasitoids emerging from a parasitised host, variation in sex-ratio of emergent parasitoids, density dependence in the parasitoid population, the time taken for an emergent parasitoid to develop the capability to successfully parasitise a host and other parasitoid life history traits (Jervis, 2007). Snyder and Ives (2003) attempt to construct a more biologically realistic model incorporating various host and parasitoid life-history traits. Their stage structured matrix model considers population changes on a daily basis within a pea aphid biocontrol context. Their model is parameterised where possible using field data and considers the effect of a generalist and a specialist predator. Kwiatkowski and Vorburger (2012) also consider population changes on a daily basis but use difference equations to formulate their model. Other approaches to modelling the *Acyrthosiphon pisum-Aphidius ervi* host-parasitoid system have been presented in the published literature.

1.6 Specific aims

The first objective of this study was to characterise the presence of *H. defensa*, *S. symbiotica*, *R. insecticola*, *Rickettsia*, *Rickettsiella*, *Spiroplasma*, PAXS and the APSE bacteriophage in all pea aphid lines held at The James Hutton Institute and to genotype these pea aphid cultures for the first time using established molecular methods. The second objective of this study was to assess the susceptibility of pea aphid lines harbouring the protective secondary endosymbiont *H. defensa* and the potentially protective endosymbiont PAXS to parasitism by *A. ervi* wasps in an exposure assay, also to refine the pre-assay and post-assay pea aphid nymph rearing technique to minimise mortality due to sources other than parasitism and to analyse previously gathered survival data to look at the costs of harbouring a protective endosymbiont. The third objective of this study, running parallel to the experimental work, was development of a multi-trophic mathematical model of the dynamics of the *A. pisum*-*A. ervi* system including endosymbiont infection parameterised, where possible, using data specific to this biological study system. The final objective was to use experimental work to inform numerical simulations, to investigate the effects of varying model parameters such as endosymbiont transmission rates and the costs and benefits of endosymbiont infection to pea aphid hosts and to look at the effect this has on the overall host-parasitoid dynamics within the context of bio-control.

2 Experimental Materials and Methods

2.1 The study system

2.1.1 The pea aphid, *Acyrthosiphon pisum*

Table 2.1: Scientific classification: *A. pisum*

Kingdom:	Animalia
Phylum:	Arthropoda
Class:	Insecta
Order:	Hemiptera
Family:	Aphididae
Tribe:	Macrosiphini
Genus:	Acyrthosiphon
Species:	Acyrthosiphon pisum (Harris, 1776)

Figure 2.1: *A. pisum* recently emerged from moult

The physical characteristics and lifecycle of *A. pisum*, classified as shown in table 2.1, are described in section 1.3. Figure 2.1 shows a pea aphid just emerged

from a moult. Feeding on a wide range of host plants in the pea (Fabaceae) family, pea aphids are an Old World species (Ferrari, 2011) introduced into the New World most probably on one of its host plants brought in for agricultural use and now pea aphids are found worldwide in regions of temperate climate. Because of the importance of pea aphids for reasons decribed in section 1.3, data is gathered on the abundance of pea aphids at a regional level during the year by means of 15 suction traps emptied daily, and distributed throughout the UK including Dundee. This source of data, coordinated by Rohamsted Research, Hertfordshire, and published on the internet (Rohamsted Research, 2015), can inform research into factors affecting aphid population dynamics and to aid deployment of suitable control measures by sponsors if and when practicable and appropriate. The data recorded at each trapping location is considered representative of populations within an 80 km radius of the trap. However, there will be considerable variations in populations at ground level due to factors such as prevalence of suitable host plants.

2.1.2 The parasitoid wasp, *Aphidius ervi*

Table 2.2: Scientific classification: *A. ervi*

Kingdom:	Animalia
Phylum:	Arthropoda
Class:	Insecta
Order:	Hymenoptera
Superfamily:	Ichneumonoidea
Family:	Braconidae
Subfamily:	Aphidiinae
Genus:	Aphidius
Species:	Aphidius ervi (Halliday, 1834)

The classification of *A. ervi* is shown is table 2.2 and figure 2.2 shows an *A. ervi* female wasp aged no more than 5 d old. *A. ervi* are commercially available for biological control and offspring (and subsequent generations) of commercially sourced *A. ervi* can be reared on aphid hosts (figure 2.3) forming a stock culture of wasps for host-parasitoid experimental work.

Figure 2.2: *A. ervi* parasitoid wasp

Figure 2.3: *A. pisum* mummy parasitised by *A. ervi* parasitoid wasp prior to emergence of next generation wasp.

Originating from the palearctic ecozone and closely associated with *A. pisum*, *A. ervi* has an extensive natural geographic range spanning Europe, North Africa, Middle East and the Far East including China, Russia, India and Northern Japan. After introduction there as a means of biological control of aphid populations, the range of *A. ervi* also now encompasses North and South America, New Zealand and Australia.

2.2 Insect cultures

2.2.1 Pea aphid clonal lines

At the start of this study, 16 *A. pisum* were kept in culture at The James Hutton Institute. Although previous cultures of some of these lines had been held at JHI, all lines were lost during the summer of 2013 due to a temperature malfunction in the insect rearing room. Replacement cultures were sourced from Imperial College, London, and The University of York. Further clonal lines were established from pea aphids collected locally to JHI. To ensure the integrity of the clonal lines, all cultures (including duplicates where applicable) were screened for the presence of secondary symbionts. Further, all lines were genotyped for the first time.

Table 2.3: Aphid clonal line information

Aphid clonal line	Origin	Collection date	Collection plant	Colour morph
JF01/29	The University of York (collected Berkshire, UK)	2001	*Lathyrus pratensis*	Green
JF200	The University of York (collected Lincolnshire, UK)	2012	*Medicago sativa*	Green
JF201	The University of York (collected Lincolnshire, UK)	2012	*Medicago sativa*	Pink
JF4500	The University of York (collected Berkshire, UK)	2010	*Lotus corniculatus*	Green
KD13/02	JHI (collected wildflower strip on site, Dundee, UK)	July 2013	Red clover	Green
KD13/04	JHI (collected Living Field on site, Dundee, UK)	July 2013	Red clover	Green
KD13/05	JHI (collected Living Field on site, Dundee, UK)	July 2013	Small white flower legume	Green
KD13/11	JHI (collected Living Field on site, Dundee, UK)	July 2013	Field bean	Green
LL01	Imperial College, London (collected Lusignan, France)	2001	*Medicago sativa*	Green
N116	Imperial College, London (collected Berkshire, UK)	2003	*Medicago sativa*	Green
N127	Imperial College, London (collected Berkshire, UK)	2003	*Medicago sativa*	Pink
N198	Imperial College, London	2003	*Medicago sativa*	Green
PS01	Imperial College, London	2001	*Vicia faba*	Green
SH1	Imperial College, London	Unknown	*Vicia faba*	Green
SH3	Imperial College, London (collected Kent, UK)	Unknown	*Vicia faba* or *Pisum Sativum*	Green
TLW03	Imperial College, London (collected Dublin, Ireland)	2003	*Medicago sativa*	Green

2.2.2 Maintenance of the pea aphid clonal lines

Figure 2.4: Stock pea aphid cultures in culture cups

Pea aphid clonal lineages were held in culture at The James Hutton Institute on pre-flowering broad bean plant cuttings, *Vicia faba* (Fabaceae; var "The Sutton"). McLean et al. (2011) report that most pea aphid clones perform successfully on this species of host plant. The *V. faba* plants were grown from seed in commercially produced insecticide free compost mix (Sinclair, UK) in a glasshouse. The compost mix contained *Sphagnum* moss peat, limestone, water-retaining gel (Celcote), initial fertiliser (Sincrostart), controlled release fertiliser (Multicote 6) and Vermiculite.

Individuals from a single clonal line were reared on plant cuttings in clear polystyrene beakers with lids with a mesh insert for ventilation (A&W Gregory and Company Limited, Kent, UK) as shown in figure 2.4. Culture cups consisted of two stacked beakers; the inner beaker with a hole of approximately 1 cm in its base. Cultures were kept in a controlled temperature insect rearing room under summer conditions at 18 °C on a 16 L: 8 D cycle. The aphids within each clonal line were genetically identical to the individuals used to establish the culture due to continued reproduction by parthenogenesis as happens naturally under summer conditions. Each culture was labelled with species name, the name allocated to the clonal line and the date that the culture (or duplicate) was established.

Cultures were maintained weekly. Each culture cup was washed and dried and the bottom cup filled, up to the level of the second cup, to a depth of approximately 1.5 cm with tap water. A fresh plant cutting was inserted into the second stacked culture cup with its stem in the water held in the first cup below. Clonal lines were

kept at low population densities in the stock cultures to maintain healthy rearing conditions. Additional duplicate cultures set up for specific experimental work were sometimes maintained at higher population densities to generate cohorts of nymphs of specific age. In the stock cultures, typically 4 to 5 pea aphid adults and 10 nymphs, if sufficient numbers allowed, were transferred with a fine paintbrush from the old plant cutting onto the new cutting. The lid was replaced and the cultures returned to the insect rearing room. Duplicates of some cultures were maintained in a controlled growth cabinet elsewhere on site.

Aphids sent from London and York had arrived by post on host plant *Pisum sativum* (see figure 2.5) and had been transferred to culture cups prepared according to this protocol. During the course of this study, further sample of clonal lines SH1 and SH3 were received.

Figure 2.5: Pea aphid samples arrived by post from Imperial College, London

2.2.3 Parasitoid wasp lines

A. ervi mummies were supplied by Koppert (Haverhill, UK). Pea aphid clonal line LL01 was selected for wasp rearing as it was free of any known secondary endosymbionts. As the parasitoids had been reared on a mix of different *A. pisum* genotypes of unknown endosymbiont infection status prior to purchase, the supplied wasps were reared for at least 1 generation on *A. pisum* clonal line LL01 before use in any experiment.

2.2.4 Wasp rearing

To maintain the parasitoid wasps in culture, a pot of 2 to 3 *V. faba* plants was placed inside a mesh cage and 15 to 20 pea aphids were transferred to the plants inside the cages and allowed to reproduce. After 1 week, about 5 previously-mated female wasps and a few male wasps were added to the infested plant cultures and allowed to forage (figure 2.6). A food source was provided for the wasps in the form of a small ball of cotton wool soaked in a $50\,\%$ v/v honey solution placed at the top of a small Eppendorf tube.

Figure 2.6: *A. pisum* infested bean plants with *A. ervi* wasps introduced

Mummies that developed on the plants from 10 days after introduction of the *A. ervi* wasps were removed carefully from the leaves using foil forceps as shown in figure 2.7. As further mummies developed, these were also removed on a daily basis. Mummies were transferred to a lidded polypropylene container with two $1\,$cm mesh-covered ventilation holes (figure 2.8). The parasitoid culture was labelled with species name, generation and date of mummy collection. Once no further mummies were observed to be developing, the infested plant material was disposed of. It was not possible to maintain parasitoid reproductive fitness beyond typically 10 generations and new wasps were purchased to re-establish the culture as required. Parasitoid cultures were reared in a growth room at $20\,^{\circ}$C on a $16\,$L: $8\,$D cycle.

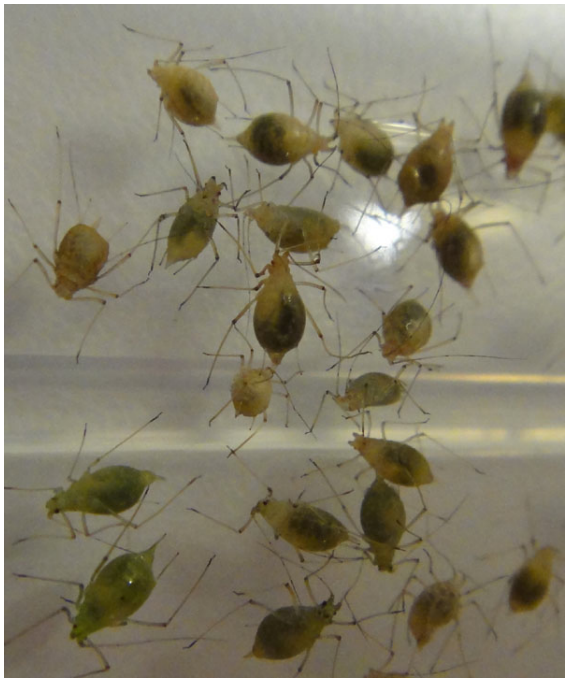


Figure 2.7: Mummies removed from plant prior to parasitoid emergence

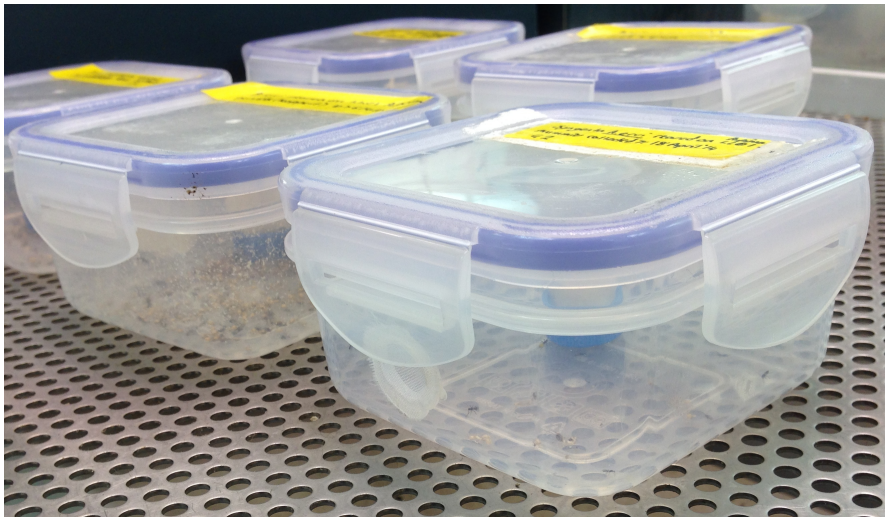


Figure 2.8: Parasitoid wasp cultures

For experimental work, emerging *A. ervi* were segregated according to date of emergence. Wasps were anaesthetised using carbon dioxide (CO_2) gas through a tube held against one of the holes in the polypropylene container whilst a finger was used to cover the other hole to prevent the gas from escaping. This gave a few minutes during which to move the wasps using a fine paintbrush into a new container. Wasps were not anaesthetised immediately prior to use in an experi-

ment. Parasitoid wasps used purely for rearing to maintain the culture were not put into day cohorts. Food was again provided in the form of a cotton wool ball soaked in $50\,\%$ v/v honey solution placed in a small plastic bottle top held in place in the bottom corner of the base of the container using Blu-Tack. Every 2 to 3 days, the cotton wool ball was washed and re-soaked in the honey solution so that the solution did not ferment.

2.3 Molecular biology methodology

2.3.1 Preparation of pea aphid DNA

2.3.1.1 Extraction of DNA from pea aphids

Using a DNeasy Blood and Tissue kit (supplied by QIAGEN Inc., Valencia, California, USA), DNA was extracted from all 16 aphid lines following a modified version of the protocol of Clarke (2009) which is based on instructions for the purification of DNA from insects using this kit (QIAGEN, 2006). The modifications were

1. the use of a number of aphids rather than $50\,$mg of insect material and
2. an additional heat step after the addition of the second lysis buffer.

The complete method was as follows. For each genotype, 8 to 10 adult aphids were placed at the bottom of an open $2\,$ml Eppendorf tube. The tube was held by the open lid and the base submerged in liquid nitrogen (N_2) for $2\,$s after which the frozen aphids were crushed in the Eppendorf tube using an ethanol sterilised micropestle and the tube was placed on ice. Next, $180\,\mu$l buffer ATL and $20\,\mu$l Proteinase K were added to the tube using Gilson pipettes. The sample was then vortexed for $15\,$s.

The Eppendorf tube was placed on a heat block (Grant QBTP Heat Block, Grant Instruments) at $55\,^{\circ}$C for a minimum of $1\,$h. During this time, the tube was removed for $15\,$s of vortexing every $15\,$min. After final removal from the heat block, the sample was vortexed for a further $15\,$s. Next, $200\,\mu$l buffer AL was added to the Eppendorf tube then the sample was vortexed again and, additionally, placed back in the heat block set at $70\,^{\circ}$C for $10\,$min.

The sample was then placed in a centrifuge (Eppendorf Desktop MicroCentifuge 5424, Eppendorf, UK) at room temperature and centrifuged for $2\,$min at $8000\,$rpm.

The supernatant was transferred by pipette to a fresh Eppendorf tube. This was done to leave the aphid debris material behind in the tube to prevent it from later clogging up the spin column. Next, $200\,\mu$l of $100\,\%$ ethanol was added to the sample. The sample was vortexed for $15\,$s.

The contents of the fresh Eppendorf tube were then transferred by pipette to a DNeasy spin column placed within a $2\,$ml collection tube. This was done carefully to ensure that the tip of the pipette did not touch the centre of the spin column. The sample was centrifuged for $1\,$min at $8000\,$rpm. The entire spin column was transferred to a fresh collection tube, following which $500\,\mu$l buffer AW1 was added to the spin column. The sample was centrifuged for $1\,$min at $8000\,$rpm. Again, the spin column was transferred to another fresh collection tube then $500\,\mu$l buffer AW2 was added to the spin column. The sample was then centrifuged for $3\,$min at $14\,000\,$rpm. Finally, the spin column was transferred to a fresh $1.5\,$ml Eppendorf tube and $200\,\mu$l buffer AE was added. After the sample was incubated at room temperature for $1\,$min and then centrifuged for $1\,$min at $8000\,$rpm, the spin column was discarded. The flow-through containing the eluted DNA was kept. Following estimation of DNA concentrations using a Spectrophotometer, aliquots of DNA ($50\,\mu$l) were transferred into $0.5\,$ml Eppendorf tubes and stored in a freezer at $-20\,^{\circ}$C.

2.3.1.2 Estimation of DNA concentrations

A Full Spectrum Ultraviolet(UV)/Visible NANODrop Spectrophotometer (ND-1000, Labtech International) was used to measure the concentration of the eluted DNA using distilled water to initialise the spectrophotometer and buffer AE for the blank sample.

2.3.2 Diagnostic Polymerase Chain Reactions (PCRs)

For diagnostic PCR screening, the *A. pisum* lines were divided into two groups. Group 1 contained lines, including some previously held, sent from Imperial College, London. Group 2 contained lines sent from The University of York and lines collected by K. Donald in 2013.

A polymerase chain reaction (PCR) generates multiple copies of a pre-selected region of a DNA molecule. DNA from all aphid lines was used as the target for amplification of gene fragments in PCR assays. The assays were performed on

an Eppendorf Mastercycler® ep gradient PCR machine (Eppendorf AG, Germany) using published primers. A script was written using the programming language R (R Core Team, 2014) to calculate reagent quantities for a given list of aphid clonal lines and to generate a layout for the PCR tubes for easy reference during the procedure.

2.3.2.1 PCR screening for *Buchnera* and bacteria other than *Buchnera* in the pea aphids

PCR amplification was used to determine the presence or absence of any bacteria in the pea aphid lines by targeting the bacterial 16S ribosomal ribonucleic acid (rRNA) gene. The primers used were the forward primer 16F27 and the reverse primer 1494R.

The 16S rRNA gene is not linked to the 23S rRNA gene by an intergenic spacer (IGS) region in *Buchnera*. This characteristic was used to differentiate between *Buchnera* and most other bacteria that do possess this gene linkage. PCR amplification was used to determine the presence or absence of any bacteria other than *Buchnera* in the pea aphid lines by amplifying this 16S-23S region. The primers used were the forward primer 10F, which targeted the I6S gene, and the reverse primer 480R, which targeted the 23S gene.

Table 2.4: Bacterial primer sequences

Bacterial infection	Primer name	Direction	5′-3′primer sequence
Any	16F27	Forward	AGAGTTTGATCMTGGCTCAG[1]
	149R	Reverse	GCTCTAGAGCGGYTACCTTGTTACGACTT[1]
Not *Buchnera*	10F	Forward	AGTTTGATCATGGCTCAGATTG[2]
	480R	Reverse	CACGGTACTGGTTCACTATCGGTC[2]

[1] Source: Lane (1991)
[2] Source: Russell et al. (2003); Sandström et al. (2001)

The PCR reaction mixture (table 2.5) was prepared in a laminar flow cabinet. All pipette tips, Eppendorf tubes, PCR tubes and lids and holding plates were UV sterilised in the cabinet prior to use. Each PCR reaction required $22\,\mu$l of reagents to which $1\,\mu$l of each primer and $1\,\mu$l of DNA were added. The reagents were GoTaq® buffer (Promega, Madison, Wisconsin, USA), Deoxyribonucleotide triphosphates

Table 2.5: Diagnostic PCR mix contents

Reagent	**Amount per reaction/ μl**
5 X Green GoTaq® reaction buffer	5.0
dNTPs (12.5 mM total)	0.5
Milli-Q ultrapure water	16.2
GoTaq® DNA polymerase	0.2
Hha 1	0.1
Forward primer (10 μM)	1.0
Reverse primer (10 μM)	1.0
DNA template	1.0
Total	**25.0**

(dNTP), molecular grade water, GoTaq® DNA polymerase (Promega, Madison, Wisconsin, USA) and *Hha 1* enzyme (Promega, Madison, Wisconsin, USA). Reagents were added as multiple quantities (calculated by the R script based on the total number of aphid clonal lines being screened) and in order of decreasing volume into a 2 ml Eppendorf tube vortexing after each addition until the *Hha 1* enzyme was added. The reagents were then mixed by drawing the reaction mix into a pipette tip 25 times. Equal volumes of reaction mix were pipetted into a smaller Eppendorf tube corresponding to each endosymbiont to be screened for. The specific forward and reverse primers were added to the reaction mix for each gene fragment to be amplified, then 24 μl was transferred into a PCR tube corresponding to each aphid clonal line being screened and a positive control and a negative control for each endosymbiont. The PCR tubes were centrifuged for 1 min at 3000 rpm.

PCR tubes were transferred to the PCR block of the thermocycler. The latter then ran for 40 min at 37 °C in order for the *Hha* 1 restriction digest enzyme to degrade any contaminating bacteria present (Vink et al., 2014), then for 10 min at 65 °C to deactivate the enzyme. Next, 1 μl of DNA template was added to each PCR tube and then the tubes were centrifuged again for 1 min at 3000 rpm. DNA from *Escherichia coli* was used as a positive control and molecular grade water was used as the negative control for 16S and 16S-23S gene fragments.

The 16S gene fragment had an expected product size of 1.5 kbit and the 16S-

23S gene fragment had an expected product size of 2.5 kbit. The PCR tubes were returned to the PCR block. Thermocyling conditions were set to an initial denaturation step of 95 °C for 2 min then 35 cycles of denaturation (95 °C for 30 s), annealing (55 °C for 30 s) and extension (72 °C for 3 min) followed by a final extension step of 72 °C for 7 min.

Lastly, PCR products were separated on an agarose gel by electrophoresis and then visualised under ultraviolet light as described in section 2.3.2.4. This separation technique used the movement of negatively charged DNA molecules through the agarose matrix with smaller fragments passing more easily through the agarose gel than large fragments.

2.3.2.2 Diagnostic PCR screening for known secondary endosymbionts in the pea aphids

PCR amplification was used to determine the presence or absence of the previously characterised secondary endosymbionts *S. symbiotica*, *H. defensa*, *R. insecticola*, *Rickettsia*, *Spiroplasma*, PAXS and *Rickettsiella*. The primers (tables 2.6 and 2.7) targeted endosymbiont specific bacterial 16S rRNA genes. The method given in section 2.3.2.1 was followed.

1. The primers for *S. symbiotica* were the forward primer 16SA1 and the reverse primer PASScmp. DNA extracted from *A. pisum* clonal line PS01 in 2012 was used as a positive control and molecular grade water was used as the negative control. The *S. symbiotica* bacterial 16S rRNA gene fragment had an expected product size of 500 bp.

2. The primers for *H. defensa* were the forward primer PABSF (differs by 1 bp) and the reverse primer 16SB1. DNA extracted from *A. pisum* clonal line JF99/04 in 2012 was used as a positive control and molecular grade water was used as the negative control. The *H. defensa* bacterial 16S rRNA gene fragment had an expected product size of of approximately 1660 bp.

3. The primers for *R. insecticola* were the forward primer U99F and the reverse primer 16SB1. DNA extracted from *A. pisum* clonal line JF98/24 (aphids frozen 2007, extracted December 2009) was used as it was known to harbour *R. insecticola* at that time and molecular grade water was used as the negative

control. The *R. insecticola* bacterial 16S rRNA gene fragment had an expected product size of 1500 bp.

The PCR amplification to determine the presence of *S. symbiotica*, *H. defensa* and *R. insecticola* bacterial 16S rRNA genes was run under the same conditions as the PCR amplification targeting the 16S rRNA and 16S-23S genes described in 2.3.2.1. For practical purposes, this often meant that all 5 PCR screens were carried out at the same time.

4. The primers for *Rickettsia* were the forward primer 16SA1 and the reverse primer Rick16SR. There was no positive control available for *Rickettsia* but the 16S rRNA gene fragment had an expected product size of approximately 200 bp. Molecular grade water was used as the negative control.

5. The primers for *Spirosplasma* were the forward primer 16SA1 and the reverse primer TKSSsp. There was no positive control available for *Spiroplasma* but the 16S rRNA gene fragment had an expected product size of approximately 500 bp. Molecular grade water was used as the negative control.

The PCR amplification to determine the presence of *Rickettsia* and *Spiroplasma* bacterial 16S rRNA genes was run with thermocyling conditions set to an initial denaturation step of $95\,^{\circ}$C for 2 min then 30 cycles of denaturation ($95\,^{\circ}$C for 1 min), annealing ($55\,^{\circ}$C for 1 min) and extension ($72\,^{\circ}$C for 2 min) followed by an final extension step of $72\,^{\circ}$C for 5 min.

6. The primers for PAXS were the forward primer PAXSF and the reverse primer 1507R. DNA extracted from *A. pisum* clonal line N116 in 2011 was used as the positive control as it was known to harbour PAXS at that time and molecular grade water was used as the negative control. The PAXS 16S rRNA gene fragment expected product size was 1057 bp.

7. The primers for *Rickettsiella* were the forward primer RCL 16S-211F and the reverse primer RCL 16S-470R. DNA extracted from *A. pisum* clonal line Bungalow Field in 2011 (then held in culture at JHI) was used as the positive control, as it was known to harbour *Rickettsiella* at that time, and molecular grade water was used as the negative control. The *Rickettsiella* gene fragment expected product size was 281 bp.

The PCR amplification to determine the presence of *PAXS* and *Rickettsiella* bacterial 16S rRNA genes was run with thermocyling conditions set to an initial denaturation step of $95\,°C$ for $2\,min$ then 35 cycles of denaturation ($95\,°C$ for $30\,s$), annealing ($55\,°C$ for $30\,s$) and extension ($72\,°C$ for $1.5\,min$) followed by an final extension step of $72\,°C$ for $5\,min$.

Finally, PCR products from all screens were separated by electrophoresis on an agarose gel and then visualised under ultraviolet light as described in section 2.3.2.4.

Table 2.6: Endosymbiont primer sequences (major endosymbionts)

Secondary endosymbiont	**Primer name**	**Direction**	**5′-3′primer sequence**
S. symbiotica	16SA1	Forward	AGAGGTTGATCMTGGCTCAG[1]
	PASScmp	Reverse	GCAATGTCTTATTAACACAT[2]
H. defensa	PABSF	Forward	AGCGCAGTTTACTGAGTTCA[3]
	16SB1	Reverse	TACGGYTACCTTGTTACGACTT[4]
R. insecticola	U99F	Forward	ATCGGGGAGTAGCTTGCTAC[5]
	16SB1	Reverse	TACGGYTACCTTGTTACGACTT[4]

[1] Source: Douglas et al. (2006); Fukatsu et al. (2000); Koga et al. (2007); Sakurai et al. (2005); Tsuchida et al. (2002). The forward primer used by Douglas et al. (2006) differed by 1 bp.
[2] Source: Douglas et al. (2006); Fukatsu et al. (2000, 2001); Koga et al. (2007); Tsuchida et al. (2002).
[3] Source: Tsuchida et al. (2002).
[4] Source: Douglas et al. (2006); Fukatsu et al. (2000); Sakurai et al. (2005); Tsuchida et al. (2002) The reverse primer used by Fukatsu et al. (2000) differed by 1 bp.
[5] Source: Darby et al. (2005); Douglas et al. (2006); Leonardo and Mondor (2006); Russell et al. (2003); Sandström et al. (2001); Tsuchida et al. (2010, 2002)

Table 2.7: Endosymbiont primer sequences (minor endosymbionts)

Secondary endosymbiont	**Primer name**	**Direction**	**5′-3′primer sequence**
Rickettsia	16SA1	Forward	AGAGGTTGATCMTGGCTCAG[1]
	Rick16SR	Reverse	CATCCATCAGCGATAAATCTTTC[6]
Spiroplasma	16SA1	Forward	AGAGGTTGATCMTGGCTCAG[1]
	TKSSsp	Reverse	TAGCCGTGGCTTTCTGGTAA[7]
PAXS	PAXSF	Forward	GAAGCAATGCAAAGAGTGTTGC[8]
	1507R	Reverse	TACCTTGTTACGACTTCACCCCAG[9]
Rickettsiella	RCL 16S-211F	Forward	GGGCCTTGCGCTCTAGGT[10]
	RCL 16S-479R	Reverse	TGGGTACCGTCACAGTAATCGA[10]

[1] Source: Douglas et al. (2006); Fukatsu et al. (2000); Koga et al. (2007); Sakurai et al. (2005); Tsuchida et al. (2002). The forward primer used by Douglas et al. (2006) differed by 1 bp.
[6] Source: Fukatsu et al. (2000, 2001); Tsuchida et al. (2002)
[7] Source: Fukatsu et al. (2001); Tsuchida et al. (2002)
[8] Source: Guay et al. (2009)
[9] Source: Guay et al. (2009); Russell et al. (2003); Sandström et al. (2001)
[10] Source: Tsuchida et al. (2010)

2.3.2.3 Diagnostic PCR screening for the APSE bacteriophage in the pea aphids

All aphid clonal lines were screened for the presence of the APSE bacteriophage using 5 sets of published primers targeting 5 APSE genes (table 2.8). Primers were supplied by Sigma-Aldrich (Gillingham, UK). The primers selected targeted bacteriophage genes P3, P35, P41, P45 and P51. The method given in section 2.3.2.1 was followed.

DNA extracted by H. Clarke from multiple aphids from the *Macrosiphum euphorbiae* aphid clonal line 10/05 on 13th June 2011, known to contain both the *H. defensa* symbiont and the APSE bacteriophage, was used as the positive control. Molecular grade water was used as the negative control.

The PCR tubes were returned to the PCR block with thermocycling conditions set to an initial denaturation step of $95\,°C$ for $2\,min$ then 11 cycles of denaturation ($94\,°C$ for $30\,s$), annealing ($56\,°C$ to $46\,°C$ for $50\,s$, touchdown) and extension ($72\,°C$ for $1.5\,min$) followed by 25 cycles of denaturation ($94\,°C$ for $30\,s$), annealing ($46\,°C$ for $50\,s$) and extension ($72\,°C$ for $1.5\,min$) then a final extension step of $72\,°C$ for $5\,min$.

After separation by electrophoresis on an agarose gel, PCR products were visualised under ultraviolet light as described in section 2.3.2.4.

2.3.2.4 Visualisation of PCR products on agarose gels

To estimate the size of the amplified DNA, PCR products were run though standard 1% agarose gels. A gel casting tray was assembled with an appropriate number of combs to give the required number of wells. Gels were made by adding UltraPureTM powdered agarose (Invitrogen/Life Technologies Ltd, Paisley, UK) to $1\,X$ Tris/Borate/EDTA (ethylenediaminetetraacetic acid) buffer (TBE) to give a $1\,X$ final concentration. Small gels were made from $0.4\,g$ agarose added to $40\,ml$ buffer; medium gels were made from $1.5\,g$ agarose added to $150\,ml$ buffer. The mixture was heated gently until the agarose crystals dissolved then, depending on the physical size of the gel, $0.5\,\mu l$ or $1.5\,\mu l$ SYBR®Safe (Life Technologies Ltd, Paisley, UK) was added. The agarose mixture was poured gently into to the gel casting tray and any air bubbles were eliminated. Pores are formed in the cooling gel with the pore size dependent on the concentration of agarose used: low concentrations of agarose yield larger pores. These pores act as a molecular sieve (Reed et al.,

Table 2.8: APSE primer sequences

APSE gene	Primer name	Direction	5′-3′primer sequence[1]
P3	APSE1.1F	Forward	TCGGGCGTAGTGTTAATGAC
	APSE2.4R	Reverse	TTCCATAGCGGAATCAAAGG
P35	APSE20.8F	Forward	GCCGCGGGGCGTGTTATTGACG
	APSE21.7R	Reverse	TTAAGGCCCGCTCATAAGCTG
P41	APSE25.0F	Forward	ATCCTGTATTGCCCGTTTTG
	APSE26.9R	Reverse	ATCATTCCGGTTACGCAAAG
P45	APSE30.6F	Forward	AGTGCAGAAGGGTAACAAAGAC
	APSE31.9R	Reverse	GGCTCTGATATTTTAGCCATGC
P51	APSE34.0F	Forward	AGGTGCGATTACCCTGTTTG
	APSE24.9R	Reverse	GATAAAACATCGCCGTTTGC

[1] Source: Degnan and Moran (2008)

2003). Once cooled and set, the gel was placed in a gel tank (H1-Set or HU25 horizontal gel units; Scie-Plas Ltd, Cambridge, UK). The tank was flooded with 1 X TBE buffer after which the gel combs were removed.

As the $5.0\,\mu$l Green GoTaq® buffer used in the PCR reactions includes a loading dye, $4\,\mu$l of PCR product from each reaction was loaded into each well. DNA ladders (Promega, Southampton, UK) of size 1 kbit and/or 100 bp corresponding the expected PCR product size were loaded into wells at the start and end of the row for each screen. To separate the products electrophoretically, small gels were run for 1 h at 60 V and medium gels for 1 h at 90 V with as much ambient light as possible excluded.

To view the resulting bands on DNA, the gel was placed on the ultraviolet filter glass stage in a FluorChem®FC2 imaging system light cabinet (Alpha Innotech Corporation, San Leandro, USA). The gel was transilluminated using UV light of wavelength 302 nm then the products visualised through a 537 nm to 540 nm green filter. A camera with associated computer software (AlphaView™) was used to capture and save an image of the gel. The size of the migrated DNA fragments on the gel was scored by comparison with the size of the migrated markers from the DNA ladder.

2.3.3 Genotyping pea aphids using microsatellite markers

Microsatellite markers, sometimes referred to as short tandem repeats (STR), are polymorphic DNA loci that consist of a repeated nucleotide sequence. In the final analysis, microsatellite loci are amplified by PCR using fluorescently labelled forward primers and unlabelled reverse primers. The resulting products are then separated according to size, in base pairs, using electrophoresis. Flourescently labelled primers are used so that, just before arriving at the positive electrode in the electrophoresis apparatus, the fluorescently labelled DNA fragments traverse the path of a laser beam causing the dyes attached to the fragments to fluoresce. The resulting light signals are separated by a diffraction system and the fluorescence is detected on a charge-coupled device (CCD) camera and converted into a digital format for import and analysis with proprietary software.

2.3.3.1 Primer selection

The first part of this study tested 13 sets of unlabelled published primers (given in table 2.9) for polymorphic loci in *Acyrthosiphon loti* and *A. pisum* using DNA templates from 5 pea aphid clonal lines (JF200, KD13/02, LL01, N116 and SH1) known to vary in their collection year and location.

The method given in section 2.3.2.1 was followed. PCR reactions were carried out in $8\,\mu$l of reaction mixture containing the same components as those used previously in the diagnostic PCRs, apart from the *Hha 1* enzyme which was not included as the microsatellite primer targets were pea aphid eukaryotic DNA and, hence, the thermocycling digest step was also omitted. The relative volumes of reaction mix are given in table 2.10. After addition of the DNA templates to the reaction mix, the PCR tubes were centrifuged for $1\,$min at $3000\,$rpm then transferred to the PCR block with thermocyling conditions set to those given in table 2.11.

PCR products were separated by electrophoresis on a thick (2%) agarose gel and viewed under ultraviolet light as described in section 2.3.2.4.

2.3.3.2 Using fluorescent primers and capillary gel electrophoresis

Five published primers ApH04M, AlC04M, ApH05M, ApF08M and AlB08M (Sigma-Aldrich, Gillingham, UK) for genotyping *A. pisum* (Caillaud et al., 2004) were used

Table 2.9: Microsatellite primer sequences

Locus	Primer name	Direction	5′-3′primer sequence[1]	Isolated from
AlA09M	AlA09MF	Forward	CCTCTCACTCCATATCTCTC	*A. loti*
	AlA09MR	Reverse	ACTTACAGTCCTCTGGCCAT	
AlB04M	AlB04MF	Forward	CAGCGCGCAGCGTATTATTA	*A. loti*
	AlB04MR	Reverse	TGGTTCGTCGTGCTGTCTCG	
AlB08M	AlB08MF	Forward	GCATGCTCGCACTCGCTTAG	*A. loti*
	AlB08MR	Reverse	CGAAATACTGCCAAAACGGG	
AlB12M	AlB12MF	Forward	GCTTAACGTCAGACGCTGAA	*A. loti*
	AlB12MR	Reverse	GCCATAACAGAGACGTCATC	
AlC04M	AlC04MF	Forward	GCCTTCCCACAGAGCTATCG	*A. loti*
	AlC04MR	Reverse	CTCGCTGTGTCCATCTTGAA	
AlC09M	AlC09MF	Forward	CGACAGTTAGCGTGCATGTT	*A. loti*
	AlC09MR	Reverse	ATCGTCACCACTACCGTCGT	
ApF08M	ApF08MF	Forward	TAATCCGTCGTAATTGCGTT	*A. pisum*
	ApF08MR	Reverse	TAAGCCCTCACTCACCCCTC	
ApG10M	ApG10MF	Forward	CAACGACGGCGGCTATACTA	*A. pisum*
	ApG10MR	Reverse	ACGAGAGCTTTCCGGCGTAT	
ApH04M	ApH04MF	Forward	CGCATCGAGTGTCGTATTAT	*A. pisum*
	ApH04MR	Reverse	GTTCCAAGGTCCTCTCTTCC	
ApH05M	ApH05MF	Forward	ACGAGAGCTTTCCGGCGTAT	*A. pisum*
	ApH05MR	Reverse	CAACGACGGCGGCTATACTA	
ApH08M	ApH08MF	Forward	GCGCACAGTGCGTATACATT	*A. pisum*
	ApH08MR	Reverse	TATTACAACGCACGTCATCG	
ApH10M	ApH10MF	Forward	ACGACGGGTGCAAGTATATT	*A. pisum*
	ApH10MR	Reverse	CAACATGACCTCGCTTCAGA	
ApH12M	ApH12MF	Forward	CTTCCACAAGAAACTCCGGT	*A. pisum*
	ApH12MR	Reverse	CTCGGTAACCACCTTGGTAG	

[1] Source: Caillaud et al. (2004)

Table 2.10: Microsatellite reaction mix contents for selecting primers

Reagent	**Amount per reaction/ μl**
5 X Green GoTaq® reaction buffer	1.6
dNTPs (12.5 mM total)	0.16
Milli-Q ultrapure water	5.4
GoTaq® DNA polymerase	0.04
Forward primer (10 μM)	0.15
Reverse primer (10 μM)	0.15
DNA template	0.5
Total	**8.0**

Table 2.11: Microsatellite thermocycling conditions

Time	**Temperature**	**Repeat**	**Action**
2 min	94 °C	-	Initial denaturation
20 s	94 °C		Denaturation
20 s	56 °C	35 cycles	Annealing
30 s	72 °C		Extension
2 min	72 °C	-	Final extension

Table 2.12: Microsatellite reaction mix contents for genotyping pea aphids

Reagent	**Amount per reaction/ μl**
5 X Clear GoTaq® reaction buffer	1.6
dNTPs (12.5 mM total)	0.16
Milli-Q ultrapure water	5.4
GoTaq® DNA polymerase	0.04
Forward primer ($10\,\mu$M)	0.15
Reverse primer ($10\,\mu$M)	0.15
DNA template	0.5
Total	**8.0**

to amplify target sequences by PCR. This method used the same components of the reaction mix used in the diagnostic PCRs except that the buffer used was 5 X Clear GoTaq® reaction buffer with forward primers modified with the addition of a 6-Carboxyfluorescein (6-FAM) fluorophore molecule to the 5′end and the mix was assembled in the same manner (table 2.12). The same general method as section 2.3.2.1 was used but in a dark laboratory with as much light excluded as possible once the fluorescent primers were in use. Again the *Hha 1* restriction digest enzyme and the digest step were omitted as the target of the microsatellite primers was eukaryotic DNA.

Once the $0.5\,\mu$l DNA templates were added and the tubes centrifuged, a programme on the thermocycler with an annealing temperature of $56\,^{\circ}$C, (Eppendorf Mastercycler®ep; Eppendorf AG, Hamburg, Germany) was used to amplify the microsatellites. When the programme was complete, the PCR products were stored at -$20\,^{\circ}$C wrapped in aluminium foil to exclude light.

Capillary electrophoresis was used to size the resulting microsatellite products. The following product preparation procedure was carried out in a dark laboratory with as much light excluded as possible. To score the size of the microsatellite products using capillary electrophoresis, each PCR product generated was diluted to 1 part in 20 using molecular grade water. Then $1\,\mu$l of the diluted PCR product and $0.16\,\mu$l of the internal lane size standard GeneScan™ carboxy-X-rhodamine (ROX) 500 (Applied Biosystems/ Life Technologies Ltd, Paisley, UK) were suspended in $8.84\,\mu$l Hi-Di™ Formamide (Applied Biosystems/ Life Technologies Ltd,

Paisley, UK) in the wells of a Thermo-fast® non-skirted 96 well plate (ABGene Ltd, Epsom, UK). The plate was sealed, wrapped in aluminium foil, and then centrifuged briefly. The fluorescently-labelled DNA fragments were then separated by capillary electrophoresis using an ABI 3730 DNA Analyser (Applied Biosystems/ Life Technologies Ltd, Paisley, UK). ABI Prism®GeneMapperTM software v. 4.0 (Applied Biosystems/ Life Technologies Ltd, Paisley, UK) was used to analyse the fragment sizes detected in each sample.

2.3.4 DNA sequencing

This study intended to sequence the partial DNA sequence for the 16S ribosomal RNA gene of the PAXS endosymbiont in PCR products obtained from DNA samples from 4 duplicated clonal lines (1 harbouring PAXS alone; 3 clonal lines harbouring H. defensa with PAXS). It was proposed to use a MinElute PCR Purification Kit to prepare existing frozen PCR samples ready to send to the JHI sequencing service then analyse DNA sequences using GeneMapper. Resulting sequence(s) were to be deposited in GenBank. A BLASTN search of GenBank would be carried out to examine homology. The homology of these PAXS sequences would be compared with the first identified PAXS sequence (Guay et al., 2009) and similar sequences of unidentified endosymbionts in other local aphid species. Differences between the PAXS sequences of single-infected and double-infected lines would be identified as such information may yield clues as to mechanism of the effectiveness against parasitism of the *H. defensa* and/or PAXS infections.

2.4 Aphid performance experiments and parasitism assays

All statistical tests were performed using R (R Core Team, 2014) within an R Studio environment (RStudio, 2014).

2.4.1 Analysis of previously gathered survival data: uninfected, single and double infected pea aphids

Survival analysis of the results of a previous short-term performance assay assessing the survival times of pea aphid clonal lines Bungalow field, JF99/04, N116, TLW03/01, JF01S, PS01, LL01, SH3 and JF98/24 showed a significant difference between these clonal lines at the 1% level with aphid line N116 harbouring *H. defensa* (with APSE) and PAXS surviving for significantly shorter times at the 5% level. Although aphids harbouring *H. defensa* (with APSE) had the lowest survivorship potential, the difference in survival between aphid endosymbiont status when grouped into the classes *H. defensa* (with APSE), *S. symbiotica* and unknown or no infection was not significant.

As part of this study, survivorship analysis using the Survival package in R (Therneau, 2014) was carried out to assess differences in this previously gathered data in survival between aphid endosymbiont status when the aphids were grouped into 4 classes according to endosymbiont infection status (*H. defensa* (with APSE), double infection of *H. defensa* with PAXS, *S. symbiotica* and unknown or no endosymbiont).

Survivorship plots were constructed for the 4 infection classes using the Kaplan-Meier estimate of survivor function which shows the probability of an individual surviving longer than time, t. This method takes into account the removal of individual aphids from the observations. The day of death of any individuals was recorded as an event and given a value of one. Aphids that stayed alive until the end of the period of time equal to the pre-reproductive period were assigned a value of zero. Aphids that became unavailable to the study either through death or loss were censored (Kaplan and Meier, 1958). A survivorship plot using Kaplan-Meier estimates, a non-parametric method of inferring the survivor function, was generated using the survfit function to show how the proportion of individuals of each endosymbiont class still alive varied with time in the experiment. The null hypothesis was that there was no difference in aphid survivorship between aphid endosymbiont status; the alternative hypothesis was that at least one pair of aphid endosymbiont classes differed significantly in aphid survivorship.

The survdiff function was used to compare the effects of weighting differences in survivorship at earlier and at later survival times and to determine whether there was any evidence against a null hypothesis of no difference in aphid sur-

vivorship between endosymbiont infection classes. Setting the argument of the survdiff function $rho = 0$ enabled a log-rank test to be carried out which weighted difference in survivorships at later survival times more heavily than differences at earlier survival times. Setting the argument $rho = 1$ enabled a Gehan-Wilcoxon test to be carried out that weighted differences in survivorship at early survival times more heavily than differences at later survival times.

The survreg function was used to fit a Weibull distribution model to the Kaplan-Meier survival estimate data using accelerated failure time parameterisation to determine significant differences in survival.

2.4.2 Rearing pea aphids in dishes

Generating cohorts of *A. pisum* nymphs of a specific age for parasitism assays and rearing attacked nymphs from parasitism assays had been previously carried out on *V. faba* cuttings in culture cups. The disadvantages of using a culture cup were that it could be time consuming to locate young nymphs on the cutting without removing the infested cutting from the culture cup, during which aphids may drop from the leaves, and an increased need to move nymphs between culture cups and experimental assays plates during which nymphs may die either during transfer or through failure to stay on the leaf after transfer (*pers. obs.*).

An experimental set-up for parasitism assays in arenas was developed at JHI by H. Clarke that fixed bean leaves onto agarose gel in petri dishes. *A. pisum* clonal line N116 was found to reproduce successfully in dishes prepared in this manner when the dish was inverted so that the leaves were on the lid. The N116 aphids generally preferred to be upside down on the lid of the dish and would often stray from the leaves and die (*pers. obs.*) if the leaves were on the bottom surface of the dish only.

2.4.2.1 Materials and methods

Petri dishes were prepared by fixing bean leaves onto agarose gel on both the top and bottom parts of the dish as shown in figure 2.9. Each part of the dish required 20 ml water in a conical flask to which 0.3 g agarose was added prior to heating until all crystals dissolved. The gel was poured into the dish and allowed to cool for approximately 2 minutes before the leaves were placed with the leaf lowerside uppermost onto the gel to cover the surface of the dish and pushed down gently

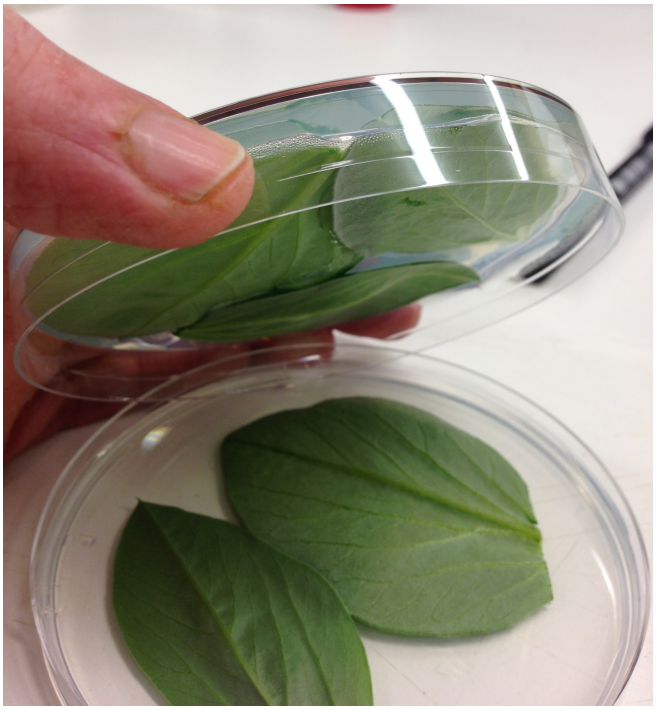


Figure 2.9: Experimental set-up for rearing aphids in dishes

onto the gel to ensure that the entire lower surfaces was in contact with the gel. Once cooled, the lid of the petri dish was replaced. Any overlap between leaves was sealed with a thin layer of agarose gel.

2.4.2.2 Measuring the time to reach adulthood and time to reach reproduction for *A. pisum* in dishes

The aim of this study was to test to see if rearing aphids in dishes was viable and to gather data on the time to reach adulthood and reproduction in growth room conditions.

Pea aphid clonal line LL01 was selected for this experiment as it is uninfected with any known secondary endosymbionts. Two late instar nymphs were transferred into a culture cup containing a *V. faba* cutting in the growth room at $18\,^{\circ}$C, $16\,$L: $8\,$D and left to reach adulthood and then reproduction. Once reproduction had started, all previously born nymphs were removed from the culture cup and the now adult LL01 pea aphids were left to generate nymphs for $24\,$h then the adults were removed from the cup. Petri dishes were prepared according to the method given in section 2.4.2.1 and 2 to 3 nymphs were transferred into each

dish. Dishes were checked daily for nymph mortality and the stage to reach each instar was noted. The day on which a nymph reached adulthood was recorded and then the day on which each adult started reproducing was recorded. Because dishes were checked daily and newly born nymphs were in very close proximity to their mother, it was possible to clearly distinguish between reproducing and non-reproducing aphids in each dish. Once reproduction was reached, each adult and its nymphs were removed from the dish.

2.4.2.3 Data analysis

The mean time to reach adulthood and mean time to reach reproduction for pea aphid clonal line LL01 under growth room conditions was calculated with associated $95\,\%$ confidence intervals.

2.4.3 Is a single infection with PAXS in pea aphids protective against parasitism by *Aphidius ervi* wasps?

It has been previously shown that pea aphids can possess one-to-several types of bacterial endosymbiont (Frantz et al., 2009). One in particular, *H. defensa*, confers resistance to parasitism by parasitoid wasps due to the presence of the APSE bacteriophage in the *H. defensa* genome (Oliver et al., 2009). Furthermore, pea aphids that harbour the dual endosymbiont infection *H. defensa* with PAXS are highly resistant to parasitism by parasitoid wasps (Guay et al., 2009). The contribution of PAXS to this increased resistance to parasitism is currently unknown. Although pea aphids harbouring a single infection of PAXS have been previously identified in the published literature (Ferrari, 2011; Henry et al., 2013), the susceptibility of such aphids to parasitoid wasps is unknown.

2.4.3.1 Preliminary assays to assess the parasitism rate of pea aphids harbouring PAXS in single and double infections

Molecular characterisation of the pea aphid lines held at JHI have identified 2 clonal lines free of any known facultative endosymbiont infection, 1 clonal line infected with *H. defensa* only, 3 clonal lines infected with *H. defensa* and PAXS and 1 clonal line infected with PAXS only.

The aim of this experiment was to carry out preliminary assays to investigate the susceptibility of pea aphids harbouring PAXS in single and double infections to parasitism by *A. ervi* wasps. Four clonal lines varying in infection status (uninfected, single infection with *H. defensa*, single infection with PAXS and double infection with *H. defensa* and PAXS) were exposed to attack by *A. ervi* parasitoid wasps and their susceptibility to parasitism assessed by calculating the proportion of aphids successfully mummified 12 d after exposure to the parasitoids.

Two hypotheses were tested. Firstly that the susceptibility to parasitism will vary due to infection with *H. defensa* between the uninfected clonal line, singly infected (with *H. defensa*) clonal line and doubly infected (with *H. defensa* and PAXS) clonal line. The null hypothesis was that there is no difference in susceptibility to parasitism between lines harbouring *H. defensa* and the uninfected line. Secondly, the susceptibility to parasitism will vary due to infection with PAXS. The null hypothesis was that there is no difference in susceptibility to parasitism between lines harbouring PAXS and the uninfected line.

2.4.3.2 Materials and methods

Four pea aphid clonal lines selected to represent the required different endosymbiont complements were used to investigate the susceptibility of pea aphids harbouring PAXS to parasitism by *A. ervi* wasps (table 2.13). Each clonal line was genotypically distinct. For each line, apterous adult and late instar aphids were transferred onto a *V. faba* cutting in a culture cup. Aphids were allowed to reproduce for 36 h. Cultures were placed in the insect rearing room and maintained at 18 °C, 16 L: 8 D. After 36 h, adult aphids were removed and nymphs left in the culture for a further 3 d by which time they were second and third instar nymphs (shown in figure 2.10)

Experimental arenas were prepared by fixing one large bean leaf onto agarose gel in petri dishes on the lower half of the dish using the procedure in section 2.4.2. Once cooled, the lid of the petri dish was replaced. For each replicate, 30 nymphs from a given pea aphid line were transferred to the arena and left to settle for 1 h (figure 2.11).

A single female *A. ervi* parasitoid wasp (assumed previously mated) aged between 2 d to 5 d old was introduced to the arena and left to forage for 2 h. Each wasp was observed for the first 5 min in the arena for each assay to make sure

Table 2.13: Pea aphid clonal lines used in parasitism assay

Pea aphid clonal line	**Facultative endosymbionts present**
LL01	None
N127	*H. defensa*
N198	*H. defensa* and PAXS
KD13/02	PAXS

Figure 2.10: Cohort of nymphs reared for parasitism assay

that she was attacking. If a wasp failed to attack within the first 5 min, she was replaced by a fresh wasp. The wasps (originally supplied by Koppert UK) were reared on *A. pisum* clonal line LL01.

Petri dishes were put in a growth room at 20 °C and 16 L: 8 D. After allowing nymphs to settle for 48 h, the aphids from each petri dish were transferred into separate culture cups containing a fresh *V. faba* cutting. The number of mummies and adult or fourth instar aphids present in each replicate were counted 12 d after initial introduction of the parasitoid wasp. This procedure was replicated 5 times using parasitoid wasps from different (subsequent) generations.

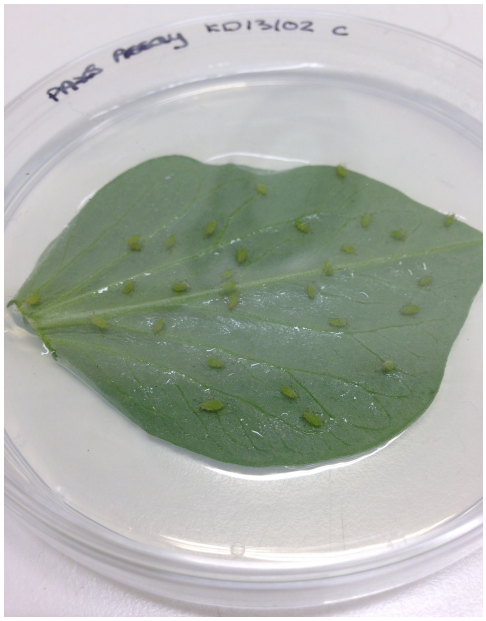


Figure 2.11: Nymphs in experimental arena ready for introduction of parasitoid wasp

2.4.3.3 Data analysis

The mean pea aphid adult survival success rate and the mean parasitoid wasp parasitism success rate with associated $95\,\%$ confidence intervals for the 4 clonal aphid lines was calculated. Analysis of variance (ANOVA) was used to investigate the differences in success rates. Replicate number was included in the analysis as a factor to account for any variation within the controlled environment rooms and/or wasp generation. Results were determined significant with a probability of $p < 0.05$.

3 Experimental Results

3.1 Characterisation of secondary endosymbionts and APSE in pea aphid lines

A set of DNA samples was extracted from the *A. pisum* stock cultures held at JHI during the period October 2013 to January 2014 following the protocol in section 2.3.1.1. The resulting concentrations from DNA extractions are shown in table 3.1. Duplicate sub-cultures of some of the pea aphid lines were maintained with some kept in a controlled growth cabinet instead of the insect rearing room to avoid complete loss of the clonal line should a problem occur with either facility. Additional sub-cultures were sometimes temporarily established for experimental work and further DNA samples extracted for screening as appropriate.

3.1.1 Presence of *Buchnera*, bacteria other than *Buchnera* and known secondary endosymbionts in the pea aphids

The infection status of the *A. pisum* clonal lines kept in the stock cultures at JHI were determined through diagnostic PCR following the procedure described in section 2.3.2. Cultures were screened for the presence of bacteria other than *Buchnera*, *Buchnera*, *S. symbiotica*, *H. defensa*, *R. insecticola*, *Rickettsia*, *Spiroplasma*, PAXS and *Rickettsiella*. PCR products were visualised on agarose gels and the results are shown in figures 3.1, 3.2, 3.3, 3.4 and 3.5. As discussed in section 3.5, the integrity of the initial stock culture of clonal line SH1 was compromised so the stock culture was restablised using a sample obtained from Imperial College, London. Screening results for the replacement SH1 culture are shown in figure 3.6 and 3.7; the "new" culture had a different complement of secondary endosymbionts present compared to the original compromised SH1 culture. The stock culture SH3 was lost during the course of this study and a replacement culture was

Table 3.1: DNA concentrations from the initial DNA extractions from stock cultures carried out during the period October 2013 to January 2014 from *A. pisum* clonal lines held in culture at JHI

Aphid clonal line	**Duplicate culture code**[1]	**DNA concentration/** $\mathrm{ng}\,\mu\mathrm{l}^{-1}$
JF01/29	-	191.7
JF200	M	87.7
	1	159.6
	2	188.9
JF201	M	6.2
	-	194.1
JF4500	-	13.6
KD13/02	1	24.3
	2	192.9
KD13/04	-	65.8
KD13/05	-	22.3
KD13/11	-	72.9
LL01	M	132.3
	1	153.1
	2	15.5
N116	-	99.1
	-	146.6
N127	-	243.3
N198	M	282.4
	1	6.9
	2	15.6
PS01	-	9.3
SH1	-	92.1
SH3	-	29.3
TLW03	-	129.6

[1] Duplicate culture locations: 1 and 2 held in insect rearing room, M held in controlled growth cabinet

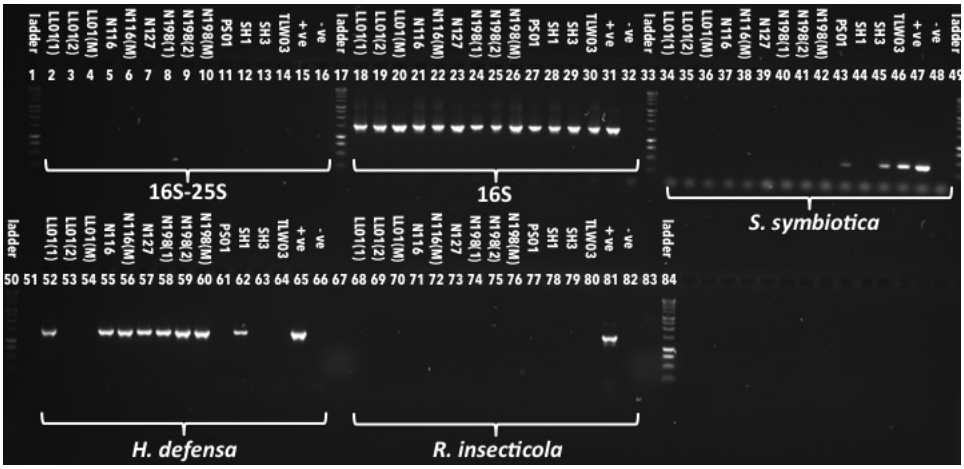


Figure 3.1: Gel electrophoresis image showing results of diagnostic PCR screen for the presence of bacteria other than *Buchnera*, *Buchnera*, *S. symbiotica*, *H. defensa* and *R. insecticola* in stock cultures of the *A. pisum* clonal lines LL01, N116, N127, N198, PS01, SH1, SH3 and TLW03 kept in culture at JHI. Promega 1 kb DNA ladder used (Promega, UK) comprising bands at 10000, 8000, 6000, 5000, 4000, 3000, 2500, 2000, 1500, 1000, 750, 500 and 250 bp. Positive and negative controls (+ve and -ve) used.

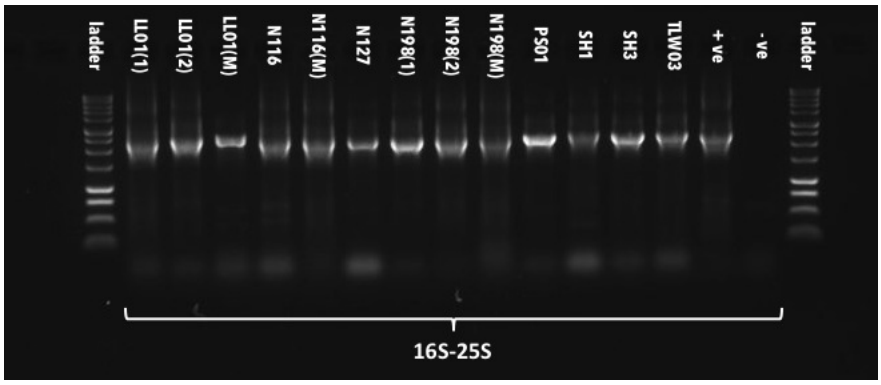


Figure 3.2: Gel electrophoresis image showing results of repeat diagnostic PCR screen for bacteria other than *Buchnera* in stock cultures of the *A. pisum* clonal lines LL01, N116, N127, N198, PS01, SH1, SH3 and TLW03 kept in culture at JHI. Promega 1 kb DNA ladder used (Promega, UK) comprising bands at 10000, 8000, 6000, 5000, 4000, 3000, 2500, 2000, 1500, 1000, 750, 500 and 250 bp. Positive and negative controls (+ve and -ve) used.

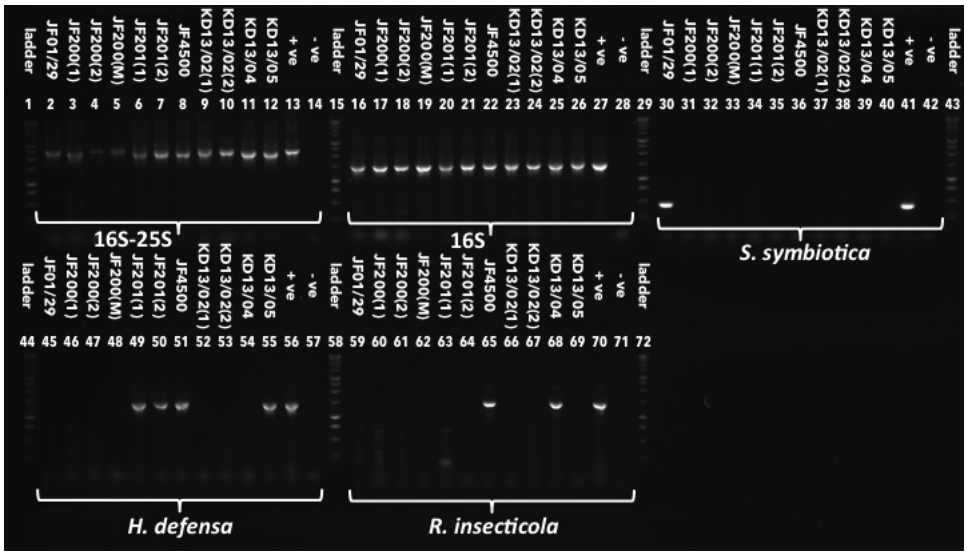


Figure 3.3: Gel electrophoresis image showing results of diagnostic PCR screen for the presence of bacteria other than *Buchnera*, *Buchnera*, *S. symbiotica*, *H. defensa* and *R. insecticola* in stock cultures of the *A. pisum* clonal lines JF01, JF200, JF201, JF4500, KD13/02, KD13/04 and KD13/05 kept in culture at JHI. Promega 1 kb DNA ladder used (Promega, UK) comprising bands at 10000, 8000, 6000, 5000, 4000, 3000, 2500, 2000, 1500, 1000, 750, 500 and 250 bp. Positive and negative controls (+ve and -ve) used.

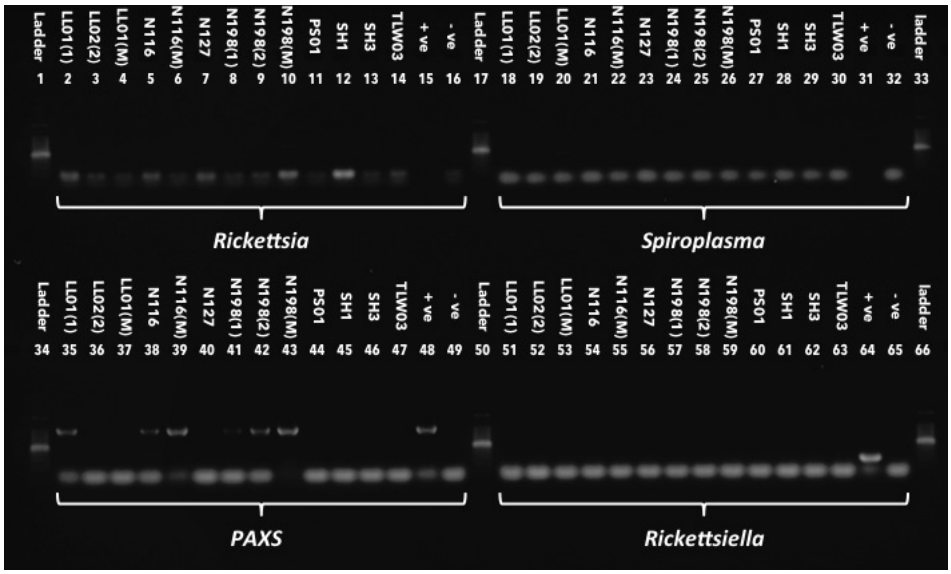


Figure 3.4: Gel electrophoresis image showing results of diagnostic PCR screen for the presence of *Rickettsia*, *Spiroplasma*, PAXS and *Rickettsiella* in stock cultures of the *A. pisum* clonal lines LL01, N116, N127, N198, PS01, SH1, SH3 and TLW03 kept in culture at JHI. Positive and negative controls (+ve and -ve) used.

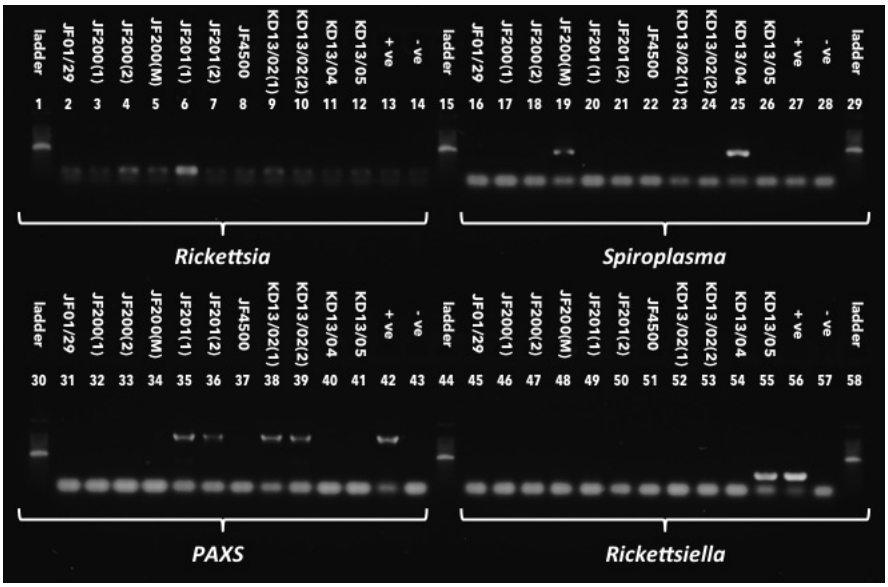


Figure 3.5: Gel electrophoresis image showing results of diagnostic PCR screen for the presence of *Rickettsia*, *Spiroplasma*, PAXS and *Rickettsiella* in stock cultures of the *A. pisum* clonal lines JF01, JF200, JF201, JF4500, KD13/02, KD13/04 and KD13/05 kept in culture at JHI. Positive and negative controls (+ve and -ve) used.

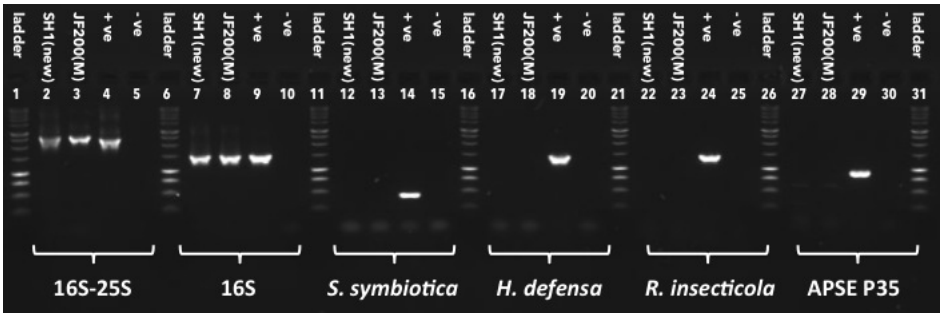


Figure 3.6: Gel electrophoresis image showing results of diagnostic PCR screen for the presence of bacteria other than *Buchnera*, *Buchnera*, *S. symbiotica*, *H. defensa*, *R. insecticola* and APSE P35 in the replacement stock culture of the *A. pisum* clonal line SH1 received to be kept in culture at JHI. Promega 1 kb DNA ladder used (Promega, UK) comprising bands at 10000, 8000, 6000, 5000, 4000, 3000, 2500, 2000, 1500, 1000, 750, 500 and 250 bp. Positive and negative controls (+ve and -ve) used.

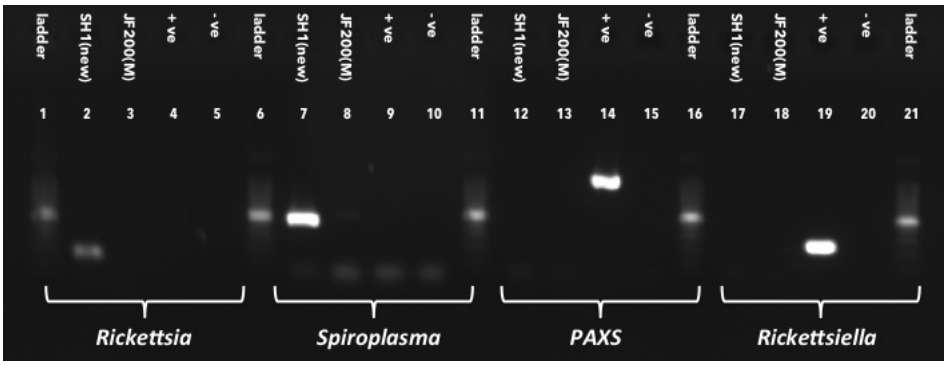


Figure 3.7: Gel electrophoresis image showing results of diagnostic PCR screen for the presence of *Rickettsia*, *Spiroplasma*, PAXS and *Rickettsiella* in the replacement stock culture of the *A. pisum* clonal line SH1 received to be kept in culture at JHI. Positive and negative controls (+ve and -ve) used.

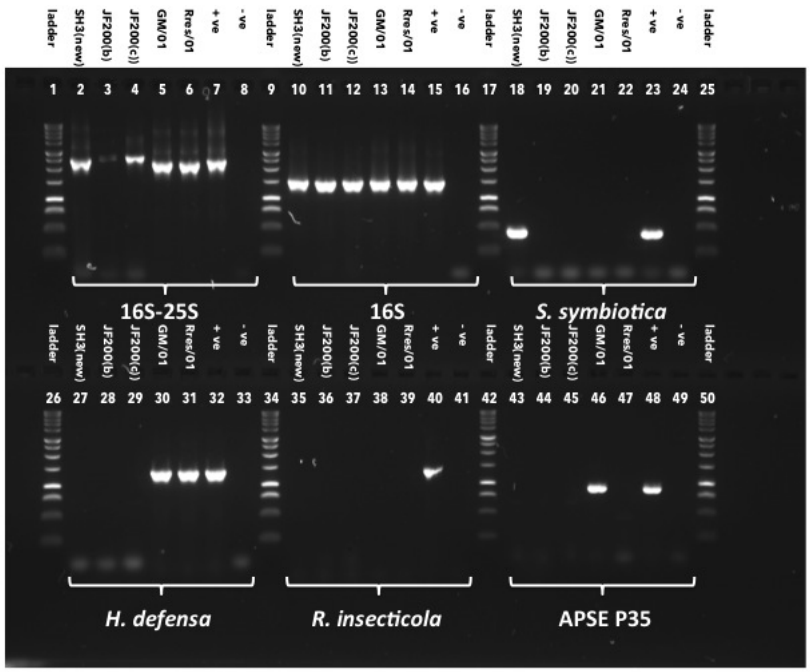


Figure 3.8: Gel electrophoresis image showing results of diagnostic PCR screen for the presence of bacteria other than *Buchnera*, *Buchnera*, *S. symbiotica*, *H. defensa*, *R. insecticola* and APSE P35 in a replacement stock culture of the *A. pisum* clonal line SH3 received to be kept in culture at JHI after the JHI stock culture died, samples of the clonal line JF200 used in a collaborative experiment on superparasitism and samples of two *M. euphorbiae* kept in culture at JHI. Promega 1 kb DNA ladder used (Promega, UK) comprising bands at 10000, 8000, 6000, 5000, 4000, 3000, 2500, 2000, 1500, 1000, 750, 500 and 250 bp. Positive and negative controls (+ve and -ve) used.

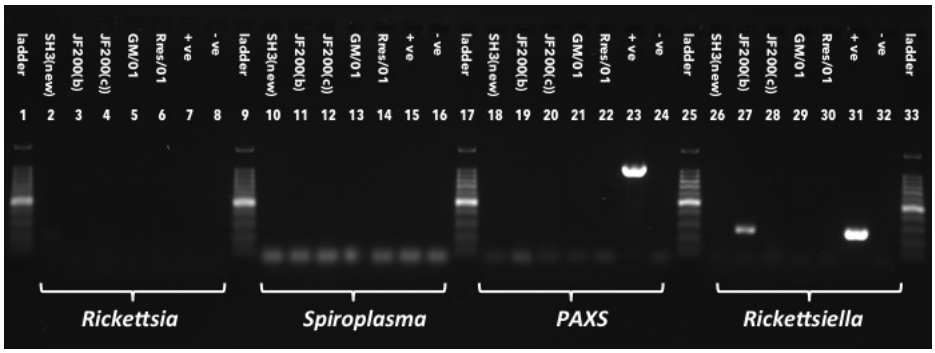


Figure 3.9: Gel electrophoresis image showing results of diagnostic PCR screen for *Rickettsia*, *Spiroplasma*, PAXS and *Rickettsiella* in a replacement stock culture of the *A. pisum* clonal line SH3 received to be kept in culture at JHI after the JHI stock culture died, samples of the clonal line JF200 used in a collaborative experiment on superparasitism and samples of two *M. euphorbiae* kept in culture at JHI. Positive and negative controls (+ve and -ve) used.

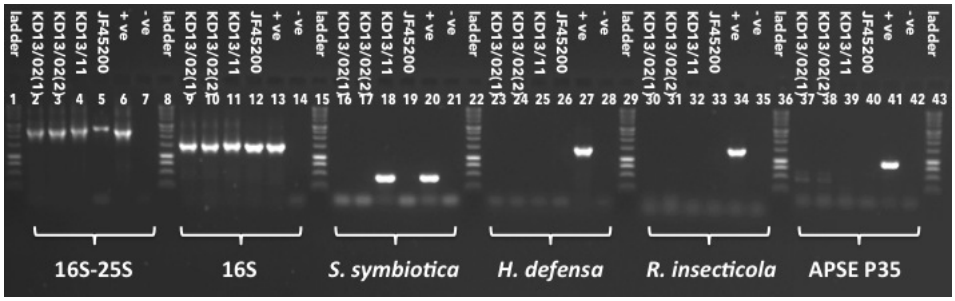


Figure 3.10: Gel electrophoresis image showing results of diagnostic PCR screen for the presence of bacteria other than *Buchnera*, *Buchnera*, *S. symbiotica*, *H. defensa*, *R. insecticola* and APSE P35 in a frozen sample of a previous stock culture of the *A. pisum* clonal line JF45200, clonal line KD13/02 again and the first screening of clonal line KD13/11. Promega 1 kb DNA ladder used (Promega, UK) comprising bands at 10000, 8000, 6000, 5000, 4000, 3000, 2500, 2000, 1500, 1000, 750, 500 and 250 bp. Positive and negative controls (+ve and -ve) used.

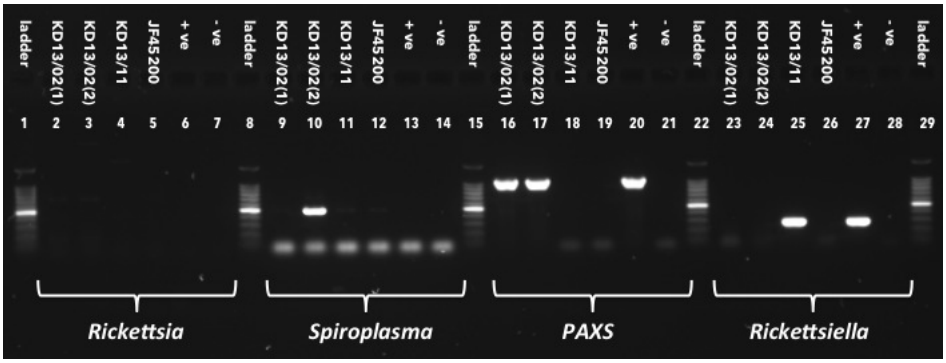


Figure 3.11: Gel electrophoresis image showing results of diagnostic PCR screen for *Rickettsia*, *Spiroplasma*, PAXS and *Rickettsiella* in a frozen sample of a previous stock culture of the *A. pisum* clonal line JF45200, clonal line KD13/02 again and the first screening of clonal line KD13/11. Positive and negative controls (+ve and -ve) used.

also sourced from Imperial College, London. Screening results for the replacement SH3 culture are shown in figure 3.8 and 3.9; the "new" culture had the same complement of secondary endosymbionts present as the original SH3 culture.

All known pea aphid secondary endosymbionts were identified in the clonal lines. Of particular note relating to work on parasitoid resistance are two endosymbiont free lines (clonal lines LL01 and JF200), one line infected with *H. defensa* only (clonal line N127), three lines with the double infection *H. defensa* and PAXS (clonal lines N116, N198 and JF201) and a single line infected with just PAXS (clonal line KD13/02). There were a variety of other single and multiple infections as summarised in table 3.2.

Because of the importance of the finding that pea aphid clonal line KD13/02 had only a single infection of PAXS present as opposed to PAXS occurring in a double infection with *H. defensa*, a further DNA sample was extracted from each KD13/02 sub-culture. Each KD13/02 DNA sample was re-screened for the presence of secondary endosymbionts (shown in figures 3.10 and 3.11) and, again, PAXS was found to be present in both sub-cultures in the absence of *H. defensa*. A transient infection with *Spiroplasma* in at least one of the pea aphids used for DNA extraction was identified in one of the KD13/02 sub-cultures shown in figure 3.11. Figures 3.10 and 3.11 show the diagnostic PCR screening results for the stock culture KD13/11 and results for a previously held stock culture JF45200. Figure 3.9 shows a likely transient infection of a pea aphid sample from a small sample of a sub-culture of clonal line JF200 (stock culture previously shown to be

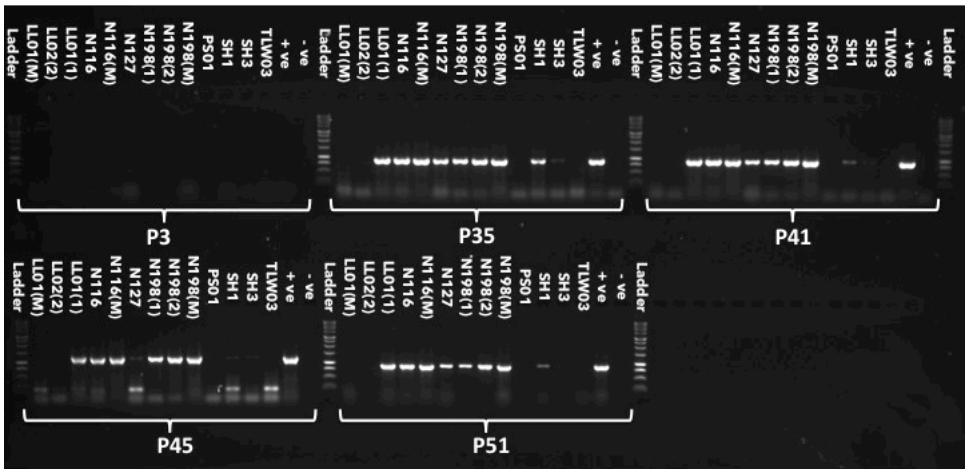


Figure 3.12: Gel electrophoresis image showing results of diagnostic PCR screen for the presence of APSE bacteriophage genes P3, P35, P41, P45 and P51 in stock cultures of the *A. pisum* clonal lines LL01, N116, N127, N198, PS01, SH1, SH3 and TLW03 kept in culture at JHI. Positive and negative controls (+ve and -ve) used.

free of known endosymbiont infection) with a *Rickettsiella*-type bacteria.

3.1.2 Presence of the APSE bacteriophage in the pea aphids

As shown in figures 3.12 and 3.13, all pea aphids lines at JHI infected with *H. defensa* also have APSE present, as summarised in table 3.2. Five primers targeting different APSE genes were tested and the primers targeting gene P35 was found to give consistent results across all pea aphid lines harbouring *H. defensa*. The primers for APSE P35 were the forward primer APSE20.8F and the reverse primer APSE21.7R (sequences in table 2.8, (Degnan and Moran, 2008)). Whilst diagnostic PCR showed positive results for the other 4 genes targeted in all *H. defensa* lines, the brightness of the lines when DNA products were viewed on an agarose gel varied between clonal lines. Although less highly conserved genes showed variation between pea aphid pines, there was consistency within duplicate cultures of the same aphid line. There was no amplified product for the primers for the P3 gene in any clonal line which is suggestive of a problem with the primer preparation. It was hence determined that screening for the P35 gene of the APSE bacteriophage was sufficient as an initial diagnostic test for the presence of APSE in the pea aphid lines.

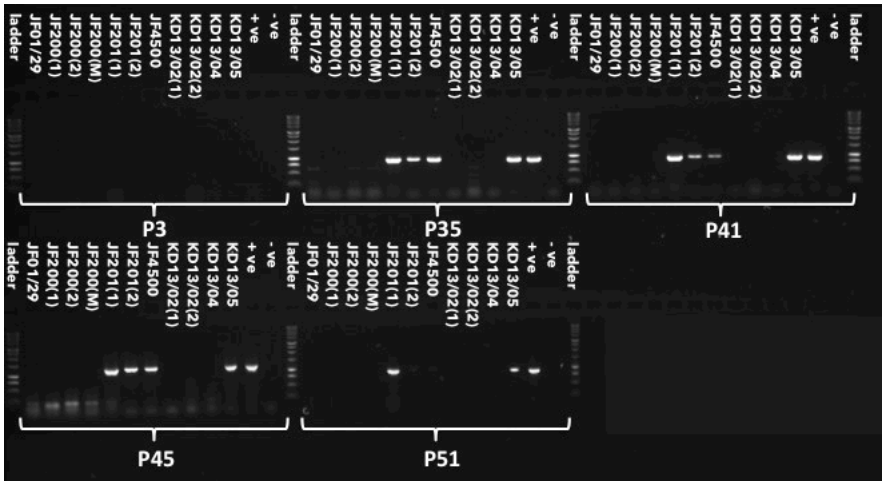


Figure 3.13: Gel electrophoresis image showing results of diagnostic PCR screen for the presence of APSE bacteriophage genes P3, P35, P41, P45 and P51 in stock cultures of the *A. pisum* clonal lines JF01, JF200, JF201, JF4500, KD13/02, KD13/04 and KD13/05 kept in culture at JHI. Positive and negative controls (+ve and -ve) used.

Table 3.2: Summary of diagnostic PCR screening for known secondary endosymbionts showing the presence or absence of infections in the stock cultures of *A. pisum* clonal lines kept in culture at JHI

	Infection status							
Pea Aphid Line	*S. symbiotica*	*H. defensa*	APSE	*R. insecticola*	*Rickettsia*	*Spiroplasma*	PAXS	*Ricketsiella*
JF200	×	×	×	×	×	×	×	×
JF45200*	×	×	×	×	×	×	×	×
LL01	×	×	×	×	×	×	×	×
N127	×	✓	✓	×	×	×	×	×
JF201	×	✓	✓	×	×	×	✓	×
N116	×	✓	✓	×	×	×	✓	×
N198	×	✓	✓	×	×	×	✓	×
JF4500	×	✓	✓	✓	×	×	×	×
KD13/05	×	✓	✓	×	×	×	×	✓
KD13/04	×	×	×	✓	×	✓	×	×
SH1	×	×	×	×	✓	✓	×	×
KD13/02	×	×	×	×	×	×	✓	×
JF01/29	✓	×	×	×	×	×	×	×
PS01	✓	×	×	×	×	×	×	×
SH3	✓	×	×	×	×	×	×	×
TLW03/01	✓	×	×	×	×	×	×	×
KD13/11	✓	×	×	×	×	×	×	✓

* line previously in culture, DNA extracted from line tested

3.2 Genotypic analysis of pea aphid lines

Fifteen sets of primers for microsatellite markers were tested (figures 3.14 and 3.15) and 5 sets of primers were identified for genotyping the pea aphid lines using fluorescent primers with the products scored using capillary electrophoresis. All pea aphid lines were screened and the size of the alleles at each microsatellite locus was found using GeneMapper.

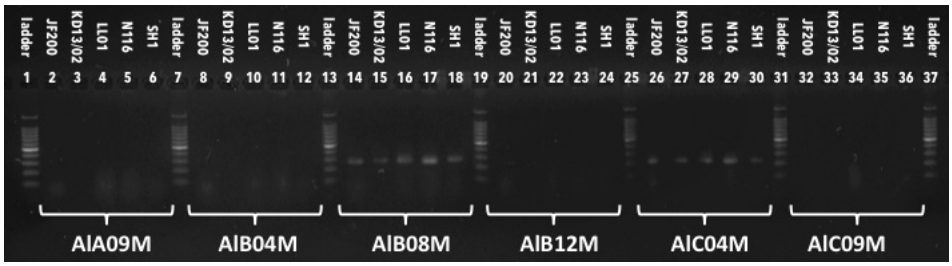


Figure 3.14: Gel electrophoresis image showing results of diagnostic PCR screen for the presence of microsatellite markers AlA09M, AlB04M, AlB08M, AlB12M, AlC04M and AlC09M in samples of the *A. pisum* clonal lines JF200, KD13/02, LL01, N116 and SH1 kept in culture at JHI

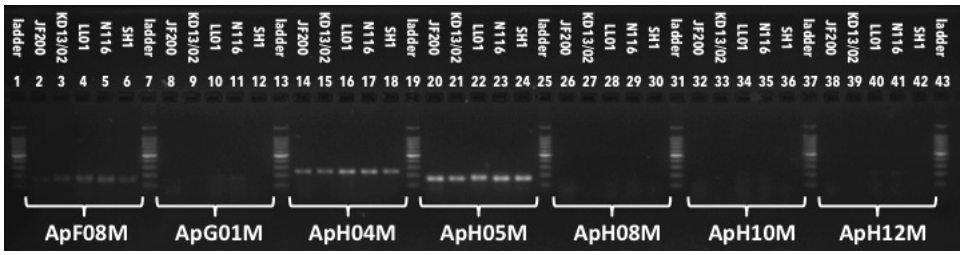


Figure 3.15: Gel electrophoresis image showing results of diagnostic PCR screen for the presence of microsatellite markers ApF08M, ApG01M, ApH05M, ApH08M, ApH10M and ApH12M in samples of the *A. pisum* clonal lines JF200, KD13/02, LL01, N116 and SH1 kept in culture at JHI

3.2.1 Genemapper results

An example of output from GeneMapper (annotated with peak size(s)) for the 5 microsatellite alleles for pea aphid clonal line N116 is shown in figures 3.16 and 3.17.

An example of output from Genemapper that is harder to interpret due to close superposition of poorly resolved allele signals is shown is figure 3.18.

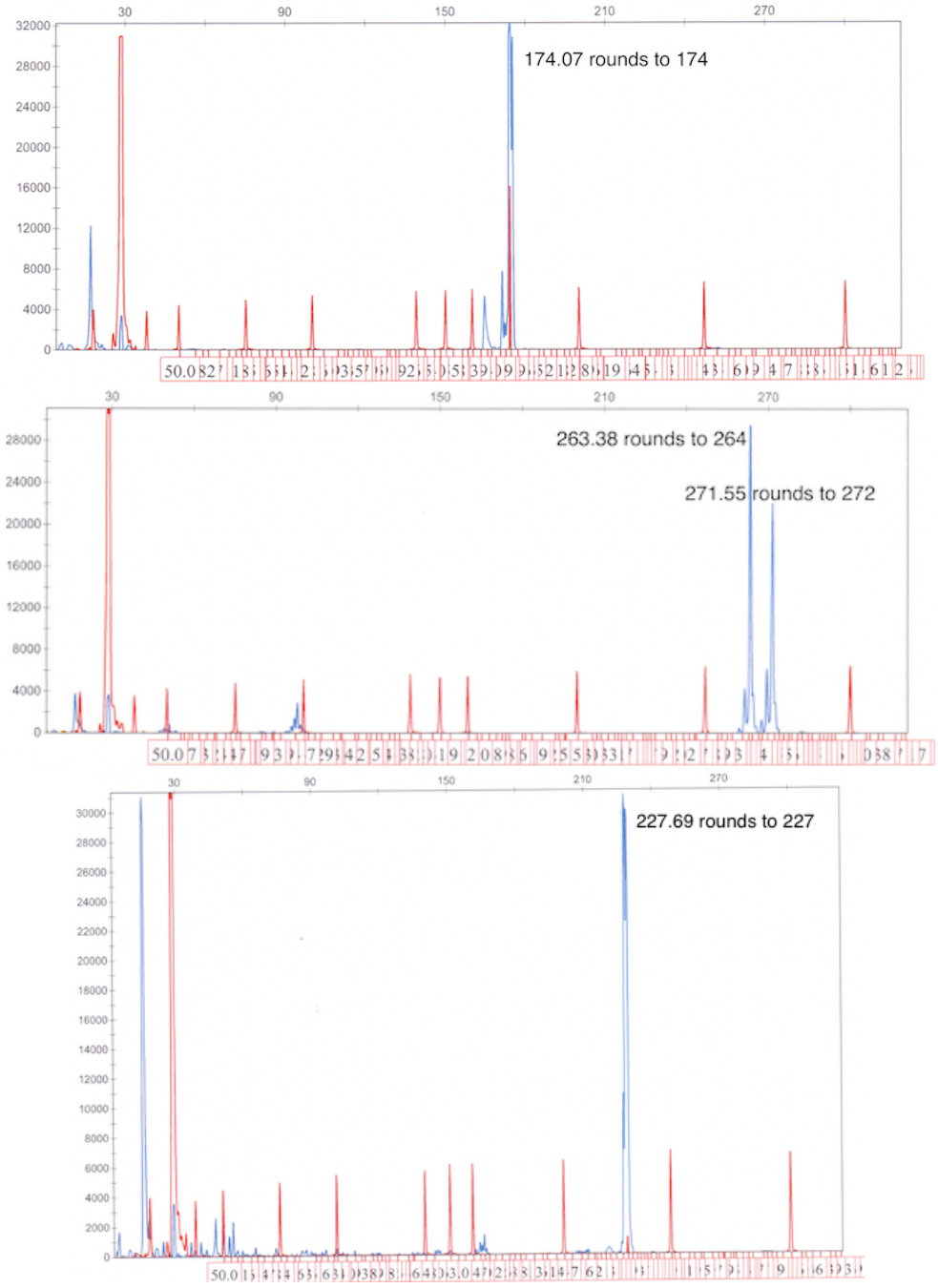


Figure 3.16: Genemapper results for microsatellite markers ApH05 (top), AiB08M (middle) and AiC04M (bottom) in *A. pisum* clonal line N116

3.2.2 Genotypic classification of pea aphid clonal lines

All pea aphid lines held at JHI are genotypically distinct (table 3.3). There was no variation between clonal lines at the ApH04M locus. Clonal line JF01/29 differs

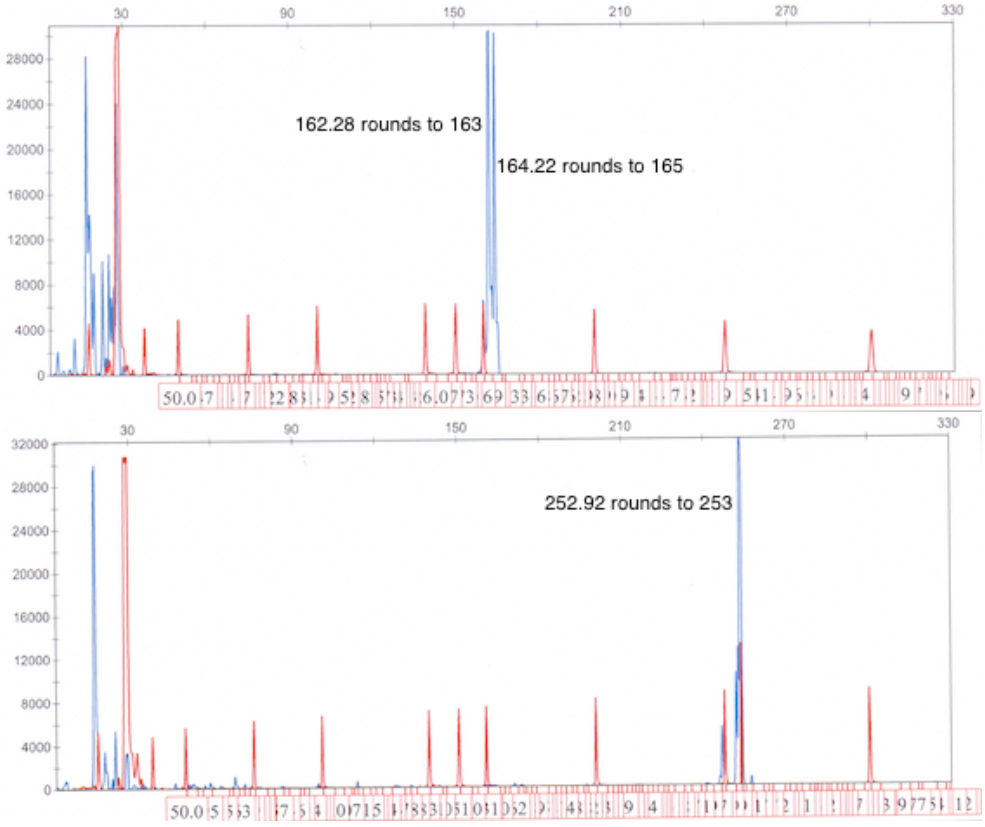


Figure 3.17: Genemapper results for microsatellite markers ApF08M (top) and ApH04M (bottom) in *A. pisum* clonal line N116

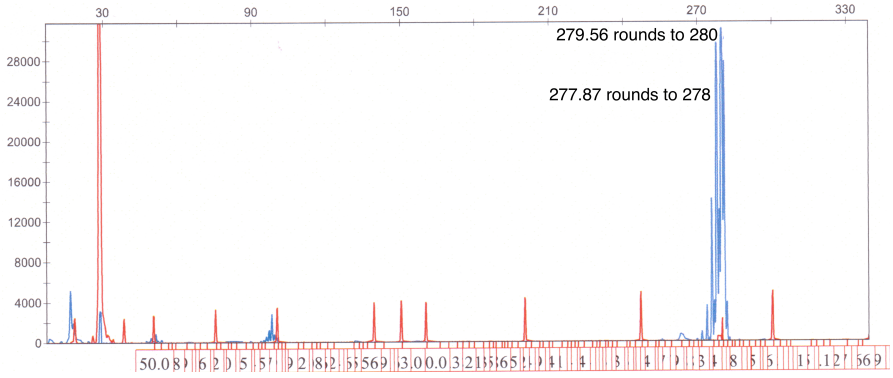


Figure 3.18: Genemapper results for microsatellite marker AlB08M in *A. pisum* clonal line KD13/02

from line PS01 by just 1 allele at the AlB08M locus and differs from line SH3 by just one allele at the ApH05M locus. All other lines are different from each other at at least 2 loci. The number of alleles found at the AlB084M and ApF08M loci was greater than the expected number of alleles indicated by the work of Caillaud

et al. (2004). The range of allele sizes found in the clonal lines used in this study was wider than that of the population from which Caillaud et al. (2004) collected their experimental samples.

Table 3.3: Pea aphid genotypes: microsatellite alleles

		Microsatellite loci:				
		ApH04M	AlC04M	ApH05M	ApF08M	AlB08M
Clonal lines:	**KD13/11**	253 253	227 227	174 174	163 163	264 270
	N116	253 253	227 227	174 174	163 165	264 272
	N198	253 253	227 227	174 174	165 165	276 278
	JF45200*	253 253	229 229	174 174	159 165	270 270
	JF200	253 253	229 229	174 174	161 163	276 278
	N127	253 253	229 229	174 174	163 163	282 286
	JF4500	253 253	229 229	174 174	163 167	272 280
	JF201	253 253	229 229	174 174	163 171	278 298
	TLW03	253 253	229 229	171 174	163 191	266 288
	KD13/02	253 253	229 229	174 174	165 173	278 280
	SH1	253 253	229 229	174 174	167 171	288 298
	KD13/04	253 253	229 229	174 177	163 165	286 288
	LL01	253 253	229 229	174 177	163 175	272 280
	KD13/05	253 253	229 229	174 177	165 165	274 284
	JF01/29	253 253	229 229	177 177	161 161	264 264
	PS01	253 253	229 229	177 177	161 161	264 284
	SH3	253 253	229 229	177 177	163 163	264 264

Table 3.4: Alleles at each microsatellite locus

Microsatellite locus:	**AlB08M**	**AlC04M**	**ApF08M**	**ApH04M**	**ApH05M**
	264	227	159	253	171*
	266*	229	161		174
	270		163		177
	272		165		
	274*		167*		
	276		171*		
	278		173*		
	280		175*		
	282		191*		
	284				
	286				
	288				
	298*				
number of alleles found	13	2	9	1	3
number of alleles expected	11	3	6	2	3
expected range alleles	266-290	229-233	163-174	256-260	174-182
size of repeat motif	2	2	2	2	3

* indicates alleles that occur only once

3.2.3 Pea aphid genotypes and secondary endosymbiont combinations

As the pea aphid clonal lines were genotypically distinct, it was not possible to test for a statistical association between overall genotype and propensity to harbour a particular endosymbiont. Table 3.5 shows just the pea aphid genotypes infected with *H. defensa* and/or PAXS only.

Table 3.5: Pea aphid genotypes with *H. defensa* and PAXS infection status

	Microsatellite loci:				
Clonal line:	ApH04M	AlC04M	ApH05M	ApF08M	AlB08M
N116	253 253	227 227	174 174	163 165	264 272
N198	253 253	227 227	174 174	165 165	276 278
N127	253 253	229 229	174 174	163 163	282 286
JF4500	253 253	229 229	174 174	163 167	272 280
JF201	253 253	229 229	174 174	163 171	278 298
KD13/05	253 253	229 229	174 177	165 165	274 284
N116	253 253	227 227	174 174	163 165	264 272
N198	253 253	227 227	174 174	165 165	276 278
JF201	253 253	229 229	174 174	163 171	278 298
KD13/02	253 253	229 229	174 174	165 173	278 280
N116	253 253	227 227	174 174	163 165	264 272
N198	253 253	227 227	174 174	165 165	276 278
JF201	253 253	229 229	174 174	163 171	278 298

indicates an *H. defensa* infected line

indicates a PAXS infected line

indicates a double-infected *H. defensa* with PAXS infected line

3.2.4 Using secondary endosymbiont screening and genotyping to maintain culture integrity

Prior to genotyping the pea aphid lines held at JHI, culture integrity was inferred from colour morph and secondary endosymbiont screening. Although direct com-

parison of pea aphids from difference clonal lines showed some visually discernible differences in appearance mainly due to size of body parts and shade, in most cases there was no other way to tell lines apart, from screening using molecular methods. As secondary endosymbiont infections can be lost in laboratory cultures or may appear on a transient basis, genotyping provides a more reliable way of checking the clonal lines. As the pea aphid lines were genotypically distinct, it was not necessary to amplify product from all 5 microsatellite loci in order to distinguish between when cultures and replacement lines were screened for the second and subsequent times.

3.3 DNA sequencing of PAXS endosymbiont

3.3.1 BLASTN search for existing PAXS 16S rDNA partial sequence

The partial DNA sequence for the 16S ribosomal RNA gene of the secondary symbiont type X of *Acyrthosiphon pisum*, PAXS, (accession number FJ821502) was deposited in GenBank after first identification as a new pea aphid secondary symbiont by Guay et al. (2009). A nucleotide-nucleotide BLAST (BLASTN) search carried out by Guay et al. (2009) showed 98% homology between this sequence for PAXS and the sequences of the 16S ribosomal RNA gene of the unidentified symbionts of the juniper aphid *Cinara juniperi* and rosa aphid *Maculolachnus submacula* deposited by Lamelas et al. (2008) (accession numbers EU348311 and EU348312 respectively). A repeat BLASTN search was carried out to see if any further sequences of interest had been deposited in GenBank since the analysis of Guay et al. (2009) and a further sequence isolated by Burke et al. (2009) from the 16S ribosomal RNA gene of the rosa aphid *M. submacula* (accession number FJ655539) was found, along with the sequences supplied by Lamelas et al. (2008), to show 99% homology with the sequence for PAXS.

3.3.2 DNA sequencing

Funding was not made available to carry out this part of the study.

3.4 Aphid performance experiments and parasitism assays

3.4.1 Analysis of previously gathered survival data: uninfected, single and double infected pea aphids

The null hypothesis was that there was no difference in aphid survivorship between aphid endosymbiont status; the alternative hypothesis was that at least 1 pair of aphid endosymbiont classes differed significantly in aphid survivorship.

A plot of the Kaplan-Meier estimates of the survival functions showed differences in aphid survival between aphid endosymbiont status. Censored aphids are indicated by the coloured vertical dashes. The difference in survival between aphid endosymbiont status (as grouped) was significant and, since $p < 0.01$, the null hypothesis was rejected at the 1% level (log-rank test: $\chi^2 = 15.8$ on 3 degrees of freedom, $p = 0.00127$; Gehan-Wilcoxon test: $\chi^2 = 14.6$ on 3 degrees of freedom, $p = 0.00221$). The value of the chi-squared test statistic was larger for the ordinary log-rank test indicating that late survival time differences are larger than early survival time differences. The log-rank test was the more appropriate test for this data. The plot (figure 3.19) shows that aphid of infection class *H. defensa* with PAXS had the lowest survivorship potential with all aphids dying before the end of the performance assay. Aphids infected with the double infection *H. defensa* with PAXS lived for a significantly shorter time than other aphid lines.

A complimentary log-log survival plot did not show a roughly linear parallel relationship for all classes. This indicates that the recorded data did not *fully* meet the assumptions of a Weibull distribution. Results from fitting a Weibull distribution model to the Kaplan-Meier survival estimate data are shown in table 3.6. The positive model coefficient estimates indicated that aphids of infection class *S. symbiotic* live longer (their survival times are more stretched out) than the reference aphid infection class *H. defensa*. The negative coefficient estimates indicated that aphid infection classes *H. defensa* with PAXS live for a shorter time (its survival time is more condensed) than the other two infection classes. This was in agreement with figure 3.19. The magnitude of the coefficients indicated the difference in survivorship between aphid lines in comparison with the reference line. The intercept is the log of the scale parameter and hence also the log of the survival rate of the reference group, whilst the log (scale) value is the reciprocal of the

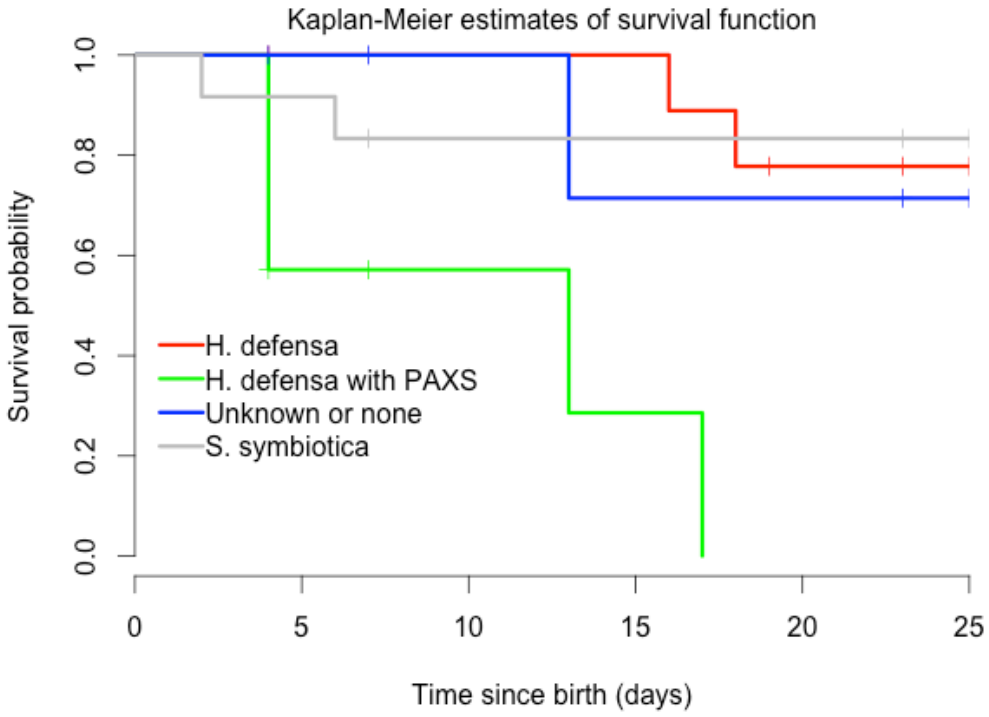


Figure 3.19: *A. pisum* survival data analysed by infection class

Table 3.6: Comparison of the significance of variation in *A. pisum* survivorship between endosymbiont infection classes

Infection class	Model coefficient	Survival ratio	P-value	Significance (level)
(Intercept)	4.128		7.98e-14	Significant (0.1%)
H. defensa		Reference		
H. defensa with PAXS	-1.788	0.1672944	0.00488	Significant (1%)
Unknown or none	-0.197	0.8211906	0.774	Not significant
S. symbiotica	0.110	1.116278	0.872	Not significant
Log(scale)	-0.378	-	0.140	Not significant

shape parameter. The scale parameter defines where the bulk of the distribution lies and the shape parameter, as suggested by the name, defines the shape of the distribution. As the model is expressed on a log scale, it is the exponent of each model coefficient that gives the effect on survival time relative to the reference group. For example, in the model, the survival time of an aphid from infection class *S. symbiotica* is increased by a factor of 1.116 compared with that of an aphid from the reference infection class *H. defensa*.

3.4.2 Rearing pea aphids in dishes

The mean time to reach adulthood for aphid clonal line LL01 reared in dishes in the growth room was 7.93 ± 0.46 d (figure 3.20). The mean time to reach reproduction for aphid clonal line LL01 reared in dishes in the growth room was 9.73 ± 0.59 d (figure 3.21).

3.4.3 Preliminary assays to assess susceptibility of single infected PAXS lines to parasitism

Figure 3.22 shows the mean mummy count with associated 95% confidence intervals for the 4 clonal lines with differing secondary endosymbiont status used in experimental arena assays to assess the susceptibility of pea aphid lines to parasitism by *A. pisum*. Analysis of variance showed a significant main effect for the clonal line factor, at the 1% level ($F_{[3,9]} = 12.281$, $p = 0.00156$); a significant main

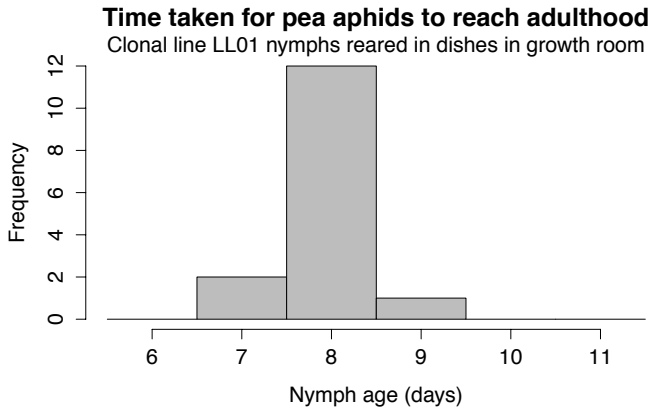


Figure 3.20: Time to reach adulthood for *A. pisum* clonal line LL01

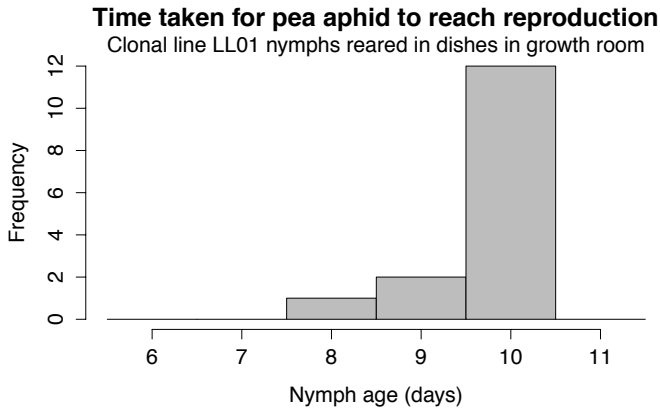


Figure 3.21: Time to reach reproduction for *A. pisum* clonal line LL01

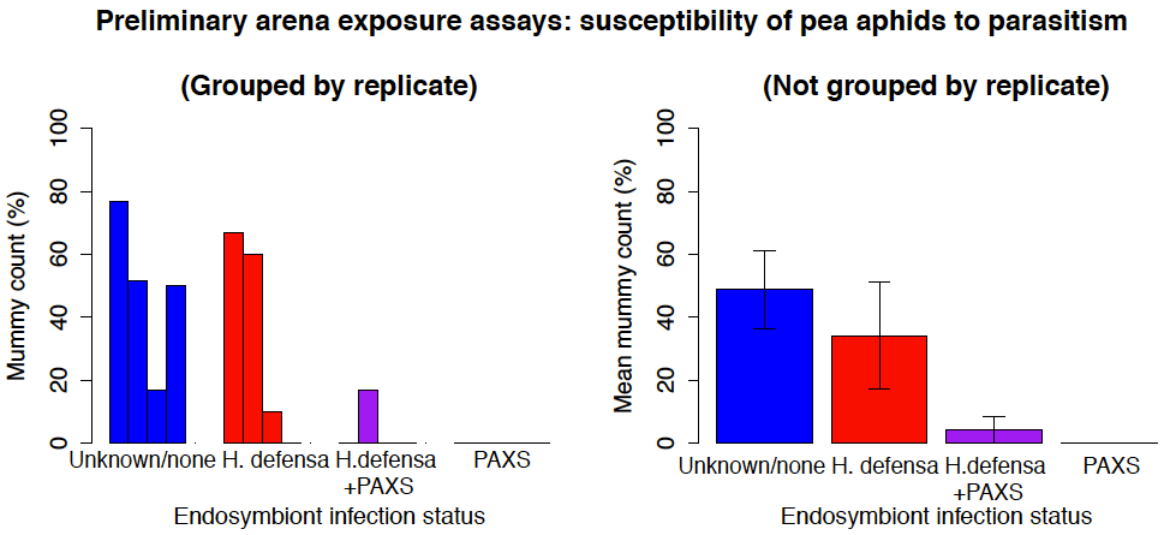


Figure 3.22: *A. pisum* mummy count for each replicate (left) and mean mummy count (right) for clonal lines LL01 (unknown/none infection), N127 (*H. defensa* infection), N198 (*H. defensa* and PAXS infections) and KD13/02 (PAXS infection) following exposure to parasitism in initial assays.

effect for the replicate factor at the 5% level, ($F_{[1,9]} = 7.585$, $p = 0.02233$); but the interaction between infection status and replicate was also significant at the 5% level, ($F_{[3,9]} = 4.092$, $p = 0.04351$). The interaction term represents the combined effect of endosymbiont infection status and replicate on the mummy count. Results show that an interaction effect is present so the impact of endosymbiont infection in these trials depends on replicate. The significance on a particular endosymbiont infection alone cannot be sensibly interpreted.

Figure 3.23 shows the mean mummy count with associated 95% confidence intervals for the 4 clonal lines used in experimental arena assays to assess the susceptibility of pea aphid lines grouped according the the presence or absence of *H. defensa*. Analysis of variance showed no significant main effect for the clonal line factor, ($F_{[1,13]} = 0.684$, $p = 0.423$); no significant main effect for the replicate factor, ($F_{[1,13]} = 2.681$, $p = 0.126$) and no significant interaction between infection status and replicate ($F_{[1,13]} = 0.127$, $p = 0.7274$).

Figure 3.24 shows the mean mummy count with associated 95% confidence intervals for the 4 clonal lines used in experimental arena assays to assess the susceptibility of pea aphid lines grouped according the the presence or absence of PAXS. Analysis of variance showed a significant main effect for the clonal line

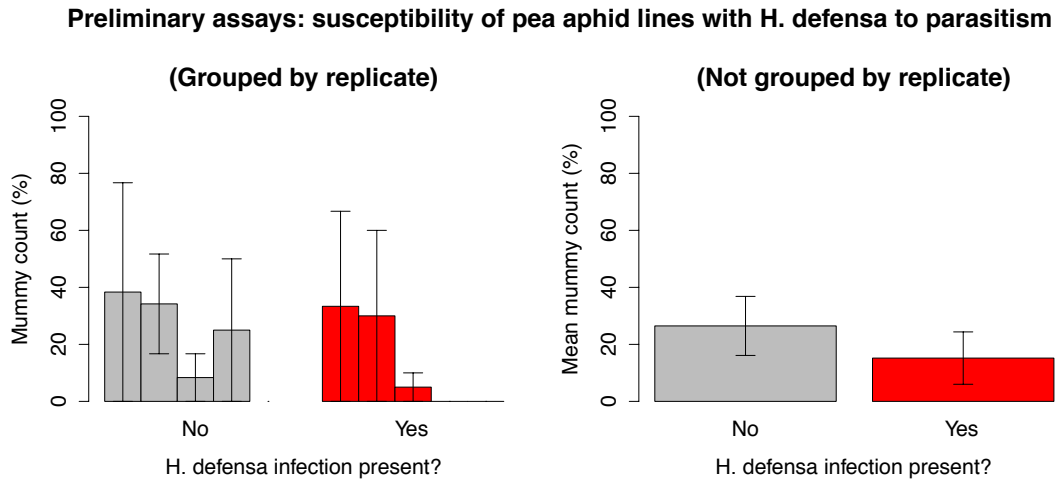


Figure 3.23: *A. pisum* mummy count for each replicate (left) and mean mummy count (right) for data from clonal lines LL01 (unknown/none infection), N127 (*H. defensa* infection), N198 (*H. defensa* and PAXS infections) and KD13/02 (PAXS infection) combined according to presence or absence of *H. defensa* following exposure to parasitism in initial assays.

factor at the 0.1% level, ($F_{[1,13]} = 23.79$, $p = 0.000302$); a significant main effect for the replicate factor at the 1% level, ($F_{[1,13]} = 11.08$, $p = 0.005435$); but the interaction between infection status and replicate was significant at the 1% level, ($F_{[1,13]} = 10.88$, $p = 0.005767$). The interaction term represents the combined effect of endosymbiont infection status and replicate on the mummy count. Results show that an interaction effect is present so the impact of endosymbiont infection in these trials depends on replicate. The significance on a particular endosymbiont infection alone cannot be sensibly interpreted.

The 2 hypotheses were tested. Firstly the hypothesis that the susceptibility to parasitism varied due to infection with *H defensa* between the uninfected clonal line, singly infected (with *H. defensa*) clonal line and doubly infected (with *H. defensa* and PAXS) clonal line was tested. There was no evidence to reject the null hypothesis which was that there is no difference in susceptibility to parasitism between lines harbouring *H. defensa* and the uninfected line. Secondly the hypothesis that the susceptibility to parasitism will vary due to infection with PAXS was tested. Due to the significant interaction between clonal line and replicate, there was insufficient evidence to reject the null hypothesis which was that there is no difference in susceptibility to parasitism between lines harbouring PAXS and the

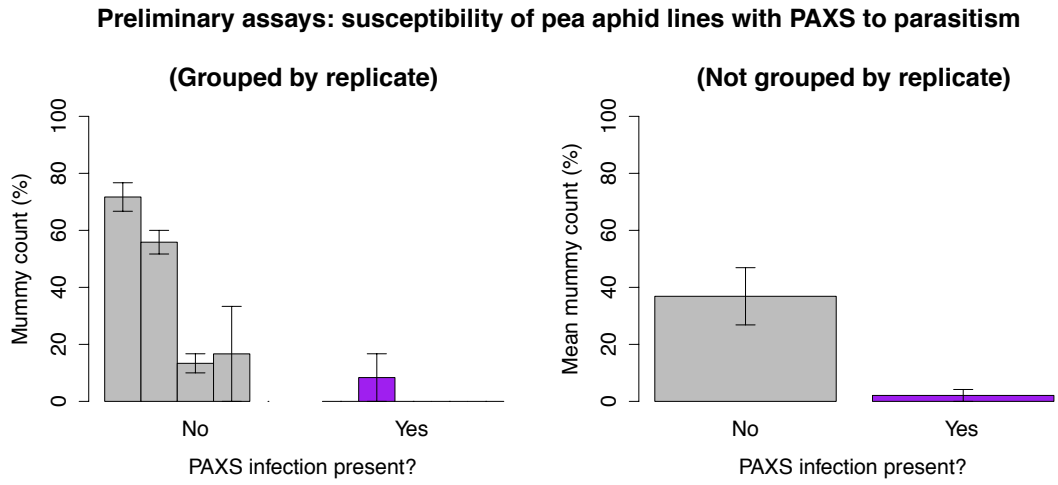


Figure 3.24: *A. pisum* mummy count for each replicate (left) and mean mummy count (right) for data from clonal lines LL01 (unknown/none infection), N127 (*H. defensa* infection), N198 (*H. defensa* and PAXS infections) and KD13/02 (PAXS infection) combined according to presence or absence of PAXS following exposure to parasitism in initial assays.

uninfected line.

3.5 Discussion

Prior to collection of pea aphid samples for DNA extraction, 6 clonal lines (JF45100, KD13/01, KD13/03, KD13/07, KD13/09 and KD13/10) were lost from culture at The James Hutton Institute due to poor culture health and opportunistic contamination of cultures with the glasshouse potato aphid *Aulacorthum solani*. Although the concentrations after DNA extraction of some resulting samples was low, all samples contained enough DNA to amplify successfully during diagnostic PCR. When carrying out DNA extractions with only a few pea aphids, the DNA concentration was generally higher if the sample was left on the heat block for 2 to 3 hours after addition of buffer ATL and Proteinase K to the sample.

When clonal line KD13/02 showed a positive result for PAXS but not *H. defensa* during diagnostic PCR, fresh samples from all duplicate cultures of KD13/02 were collected and new DNA samples extracted. These samples were then tested for the presence of PAXS and *H. defensa* and a single infection of PAXS was confirmed as far as possible from a diagnostic PCR screen. Positive results from the PCR

screen for the intergenic 16S to 23S space indicative of secondary endosymbiont infection in KD13/02 pea aphids provide further evidence for such an infection. Upon detection of PAXS as a new facultative pea aphid symbiont, Guay et al. (2009) assert that pea aphids harbouring a double infection of *H. defensa* with PAXS may often be found in the field and report that they collected pea aphids of this infection status on alfalfa and red clover host plants. After comparison of the partial 16S ribosomal DNA PAXS sequence with the sequence of the *H. defensa* genome (GenBank accession number CP001277), Guay et al. (2009) rule out the possibility of their PAXS sequence arising from an extra copy of the 16S rDNA gene in *H. defensa*. Based on the results of their BLASTN search, Guay et al. (2009) put forward the suggestion that PAXS may occur as a single infection in a range of aphids on a range of host plants. The detection of a PAXS as a single infection without *H. defensa* in pea aphid line KD13/02 at JHI provides evidence to verify these assertions. *C. juniperi* and *M. submacula*, where unidentified secondary symbionts with a high degree of homology with PAXS are known to exist without an associated *H. defensa* infection, are known pests in the UK (Alford, 2012). The female pea aphid from which clonal line KD13/02 was established was collected from a red clover host plant in July 2013 from a strip of land containing mixed wild flowers at JHI. To confirm the presence of the same PAXS infection in the KD13/02 clonal line as in the clonal lines collected by Guay et al. (2009), the DNA samples held at JHI should be sequenced. It would be interesting to sequence the PAXS sequences from all the pea aphid lines at JHI testing positive on diagnostic PCR screening for PAXS to see if the partial 16S rDNA sequences in single- and double-infected pea aphid lines are the same. A further BLASTN search using the results from the sequencing should then be carried out to assess the degree of homology between known sequences.

Transient infections of, or contamination with, a *Spiroplasma* and a *Rickettsiella*-like infection were observed in some pea aphid lines. Diagnostic PCR screening for known endosymbionts of a sample of two adults from pea aphid clonal line JF200 yielded a faint positive result but there was not a strong positive for the 16S-23S screen for this sample which would have been suggestive of a secondary endosymbiont infection. None of the pea aphid lines harboured *Rickettsiella* alone and the sample tested negative for *H. defensa* so contamination with a different clonal line can be ruled out as a source of infection. Diagnostic PCR screening of the nymphs born from the adults in the sample was negative for all known

endosymbionts. It can be inferred from this that at least 1 of the adult aphids was infected or contaminated with a *Rickettsiella*-like infection. There is no evidence in the literature that *Rickettsiella* infections are unstable in aphid cultures (Lukasik et al., 2013) but, as *Rickettsiella* is a large family and *Rickettsiella* seems to be quite a widespread entomopathogen, it is possible that the diagnostic PCR detected a surface or transient environmental *Rickettsiella*-like contaminant that is related to the species infecting pea aphids. The same infection was not observed in the JF200 stock cultures. The plant material used for aphid rearing at the time of the infection was sometimes of a poor quality (figure 3.25) and a fungal infection was affecting some of the cultures (figure 3.26). JF200 appeared particularly susceptible to the fungal infection (*pers. obs.*). It took approximately 4 months to eliminate the fungal infection from the pea aphid cultures through strict hygiene when culturing the pea aphids on a weekly basis including cleaning the paintbrush used to transfer aphids with 100% ethanol between clonal lines and even between aphid transfers within a line and avoiding transfer of aphid from a location in a culture cups when fungal spores from a nearby infected aphid may have landed. A *Spiroplasma* infection was observed in a later diagnostic PCR screening for secondary endosymbionts in pea aphid clonal line KD13/02. This was considered to be a transient infection as it was very unlikely that this was due to contamination with either of the clonal lines KD13/04 and SH1 (the only pea aphid lines kept in culture at JHI harbouring *Spiroplasma*) as both of these clonal lines also harboured other endosymbionts and, apart from PAXS, the sub-culture of KD13/02 did not yield positive diagnostic PCR results for any other endosymbiont.

APSE gene P35, responsible for injecting phage DNA into symbiont host cells, is highly conserved. APSE gene 51 is not highly conserved. Degnan and Moran (2008) assert that diagnostic PCR alone is not enough to confirm the presence of the APSE bacteriophage and that a Southern blot or amplification of intergenic spacers is essential. It was determined through testing several sets of primers, that the primers for the P35 gene provide a reliable initial diagnostic test for the presence of this variant of the APSE bacteriophage in the pea aphid clonal lines.

Capilary gel electrophoresis was used to separate DNA fragments corresponding to particular microsatellite loci. Very small DNA fragments such as these could have been separated using polyacrylamide gels (Reed et al., 2003). In capillary gel electrophoresis, the fine capillary houses a polymer through which molecules pass. As with electrophoresis carried out on agarose gels, larger DNA fragments

Figure 3.25: Appearance of poor quality *V. faba* plant material after use in stock cultures

Figure 3.26: Pea aphids from stock culture clonal line N127 with fungal infection

are slowed more by the polymer. Capillary electrophoresis is a fast, high resolution separation technique. Given the close spacing (approximately 2 bp of some alleles, it is appropriate to apply this technique here. A further advantage is that all loci can be detected and resolved in one capillary injection (Life-Technologies, 2014). Capillary gel electrophoresis had been used to genotype the potato aphid cultures at JHI (Clarke, 2013). Fewer potato aphid genotypes were found to exist in culture at JHI compared to pea aphid genotypes. This is due to the lack of a sexually reproducing generation of potato aphids in the UK, unlike pea aphids that intersperse asexual reproduction with sexual production in the field when environmental conditions deviate in autumn from the summer conditions that perpetuate clonal parthenogenic reproduction.

Superposition of closely spaced peaks in Genemapper can be problematic when the microsatellite repeat motif is very small as seen in figure 3.18. Testing further microsatellite primers should enable other loci to be identified with a longer repeat motif to reduce the degree of overlap. Alternatively, if the lack of clear peaks or split peaks in the signal is due to artefacts cause by amplification from other binding reactions, various techniques exist to improve signal quality such as modifying the thermocycling conditions, optimising the magnesium (Mg^{2+}) ion concentrations and/or "tailing" the 5′ end of the reverse primer with at least one additional nucleotide (Life-Technologies, 2014).

The 1% difference in homology between BLASTN searches (section 3.3.1) is a very small difference. In the absence of any sequence updates, an explanation for this difference is the setting used to do the alignments and the Blocks Substitution Matrix (BLOSUM) matrix used.

After initial genotyping of all pea aphid lines held in culture at JHI, only 2 lines were identical at all 5 alleles. Both lines were of pink colour morph and harboured the same secondary endosymbiont infection. As some clonal lines had been restocked from cultures held at Imperial College, London, enquiries were made as to the colour morph and infection status of the original lines held there and a DNA sample extracted from the SH1 clonal line held at JHI in 2011 was rescreened for secondary endosymbiont complement and genotyped. It became apparent that the culture at JHI, thought to be SH1, was not the original SH1 line but likely clonal line N127. A new SH1 culture was then supplied by Imperial College. Another interesting finding from the initial genotyping was that one of the LL01 cultures used at JHI for wasp rearing had been noted (K. Donald and

A. Karley, *pers. comm.*, 2013) as having very few mummies formed after exposure to parasitioid wasps and hence fewer wasps in each subsequent generation. The genotyping showed that this particular LL01 culture was contaminated with a pea aphid line harbouring *H. defensa* with PAXS previously shown to be highly protective against parasitism. The other LL01 culture held at JHI was free from contamination and used for subsequent wasp rearing. These two examples illustrate the benefits of genotyping as a means to maintain the integrity of clonal lines.

Collaborative superparasitism assays contributed to by the author of this study, using *A. pisum* and *A. ervi* insect material sub-cultured at JHI and experimental techniques developed during the course of this study, confirmed that when double-infected single-attacked pea aphids are double-attacked, wasp eggs are deposited (Donald et al., 2016).

Upon dissection of pea aphids used in the collaborative work on superparasitism, it was observed that the consistency of the fluid contained within the aphids varied with clonal line. Fluid from pea aphids from the clonal line JF200 appeared very clear when dissected allowing for rapid detection of any deposited wasp eggs, whereas fluids from pea aphids from other clonal lines were noticeably more opaque making it harder to find deposited wasp eggs. It is suggested that the difference may be due to differing fat content within the pea aphids, with aphids from clonal line JF200 having less fat in their haemocoel. Oliver et al. (2012) report that teratocytes (from extraembryonic tissues) in *A. ervi* are known to synthesise and release a fatty acid binding protein, Ae-FABP, ostensibly linked to the diversion of aphid host resources to developing wasp larvae. Further, Sabri et al. (2011) discuss the early development after *A. ervi* oviposition of a placenta-like structure within the aphid host. The parasitoid development is linked to the host fat resources. As there has been a genotypic variation in host resistance to parasitism observed between clonal lines with clonal line JF200 appearing to exhibit a high genotypic resistance to parasitism in the absence of a known secondary endosymbiont infection, it would be valuable to test a link between pea aphid fat content and early development failure of an oviposited *A. ervi* egg. The biological mechanism for this may be the lack of enough fat to support the placenta-like structure.

Following parasitism assays in a dish, it has been standard practice at JHI to transfer aphids into a culture cup containing a *V. faba* cutting which is the standard

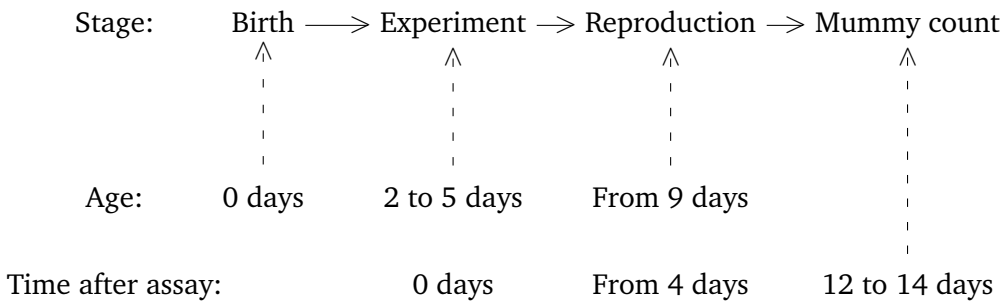
set-up for maintaining an aphid culture. In culture cups containing experimental pea aphid lines that harboured a dual-infection of *H. defensa* with PAXS (N198 and N116) or a highly resistant line, overcrowding once surviving aphid reached reproduction was a problem. After this, it was not possible to distinguish between mortality due to parasitism and mortality due to infraspecific competition. The average time for uninfected pea aphid from line LL01 to reach adulthood was 7.93 ± 0.46 d and to reach reproduction was 9.73 ± 0.59 d under growth room conditions where pea aphids are reared after parasitism. As it has been shown that pea aphids can be reared in dishes, attacked pea aphids can be left for a few days in dishes, after an assay, to reduce mortality due to transferring any pea aphids nymphs directly after attack when they would be particularly vulnerable to further stress. It would be prudent to check culture cups regularly from 4 d post-assay onwards and to remove the offspring of aphids used in the assay regularly prior to final mummy count (figure 3.27).

Using day cohorts of parasitoids for parasitism assays is particularly important at the lower end of the 2 d to 5 d old range, especially when the parasitoid line has already been held in culture for several generations. Quicke (1997) cites evidence that mated female parasitoids attack more hosts than unmated female parasitoids of the same age. Personal observation has shown that by the time that *A. ervi* parasitoid have been held in culture for more than 5 generations, the number of males emerging has highly outnumbered the female parasitoids emerging and that, when used in parasitism assays, unmated 1 d old female parasitoids attempt to oviposit noticeably less often than 2 d old mated females. However, it should be stressed that no attempt was made here to quantify these observations at part of this study. It was found to be prudent to capture the wasps used in parasitism assays individually in micro-test-tubes and to check under a microscope that the wasps used were indeed female.

3.6 Summary and Conclusions

16 pea aphid clonal lines were kept in culture at JHI. DNA was extracted from a sample of aphids from each of the clonal lines. DNA from each clonal line was used in PCR screens for the presence or absence of the obligate endosymbiont *Buchnera*, bacteria other than *Buchnera* and the known secondary endosymbionts *S. symbiotica*, *H. defensa*, *R. insecticola*, *Rickettsia*, *Spiroplasma*, PAXS and *Rickettsiella*. All

Figure 3.27: Post-assay pea aphid rearing timings



pea aphid clonal lines harboured the primary endosymbiont *Buchnera* and, of the 16 pea aphid clonal lines, 14 lines were found to harbour 1 or more of the known pea aphid secondary endosymbionts. There were 2 secondary endosymbiont free lines (clonal lines LL01 and JF200), 1 line infected with *H. defensa* only (clonal line N127), 3 lines with the double infection *H. defensa* with PAXS (clonal lines N116, N198 and JF201) and 1 line infected with single infection PAXS (clonal line KD13/02). With the exception of transient *Spiroplasma* and a *Rickettsiella*-type infections, all other infections appeared stable during the duration of this study.

Further, the 16 pea aphid lines were screened for the presence or absence of the APSE bacteriophage. APSE was found in the 6 pea aphid clonal lines also found to harbour a *H. defensa* infection. From the 5 sets of primers tested, the PCR primers targeting the P35 APSE gene were found to give consistent results across all pea aphid lines harbouring this infection and, hence, sufficiently reliable to use as an initial diagnostic PCR test.

The pea aphid lines held at JHI were genotyped using microsatellite markers for the first time. 15 sets of primers from the published literature were tested and 5 sets determined to be suitable for subsequent PCR using fluorescent primers with the size of the alleles at each microsatellite locus then scored using capillary electrophoresis. This revealed that all pea aphid clonal lines held at JHI were genotypically distinct. This degree of genotypic variation reflects the existence of a sexual generation in the field pea aphid populations in temperate regions from which these stock pea aphid cultures were originally established. There was not enough data to assess any relationship between genotype and secondary endosymbiont infection.

Survival data from a previously carried out performance assay was re-analysed

in light of the increasing interest in the high resistance to parasitism apparently conferred to pea aphids by the dual infection of the secondary endosymbionts *H. defensa* with PAXS. The null hypothesis was that there was no difference in aphid survivorship between aphid endosymbiont status; the alternative hypothesis was that at least one pair of aphid endosymbiont classes differed significantly in aphid survivorship. A plot of the Kaplan-Meier estimates of the survival functions showed differences in aphid survival between aphid endosymbiont status. The difference in survival between aphid endosymbiont status (when grouped as *H. defensa*, *H. defensa* with PAXS, unknown or none, *S. symbiotica*) was significant and, since $p < 0.01$, the null hypothesis was rejected at the 1% level. Aphids infected with the double infection *H. defensa* with PAXS lived for a significantly shorter time than other aphid lines.

A new experimental method of rearing pea aphids in petri dishes rather than culture cups was trialled successfully. The time for pea aphids to reach adulthood and the time for pea aphids to reach reproduction was measured for pea aphids reared in petri dishes. The average time for uninfected pea aphid from line LL01 to reach adulthood was 7.93 ± 0.46 d and to reach reproduction was 9.73 ± 0.59 d under growth room conditions where pea aphids are reared after parasitism.

Upon discovery of a pea aphid line harbouring a single secondary endosymbiont infection of PAXS without *H.*defensa, preliminary assays were carried out to assess the susceptibility to parasitism by *A. ervi* parasitoid wasps of pea aphids harbouring single infections of PAXS and dual infections of PAXS with *H. defensa*. Although mummy counts for PAXS infected pea aphid lines were lower, statistical analysis suggested that there was no evidence to reject the null hypothesis which was that there is no difference in susceptibility to parasitism between lines harbouring *H. defensa* and the uninfected line used in the assay. Further, when the hypothesis that the susceptibility to parasitism will vary due to infection with PAXS was tested, there was insufficient evidence to reject the null hypothesis was that there is no difference in susceptibility to parasitism between lines harbouring PAXS and the uninfected line because there was a significant interaction between clonal line and replicate. Further assays are necessary before any firm conclusion can be reached.

4 Modelling endosymbiont-mediated protection

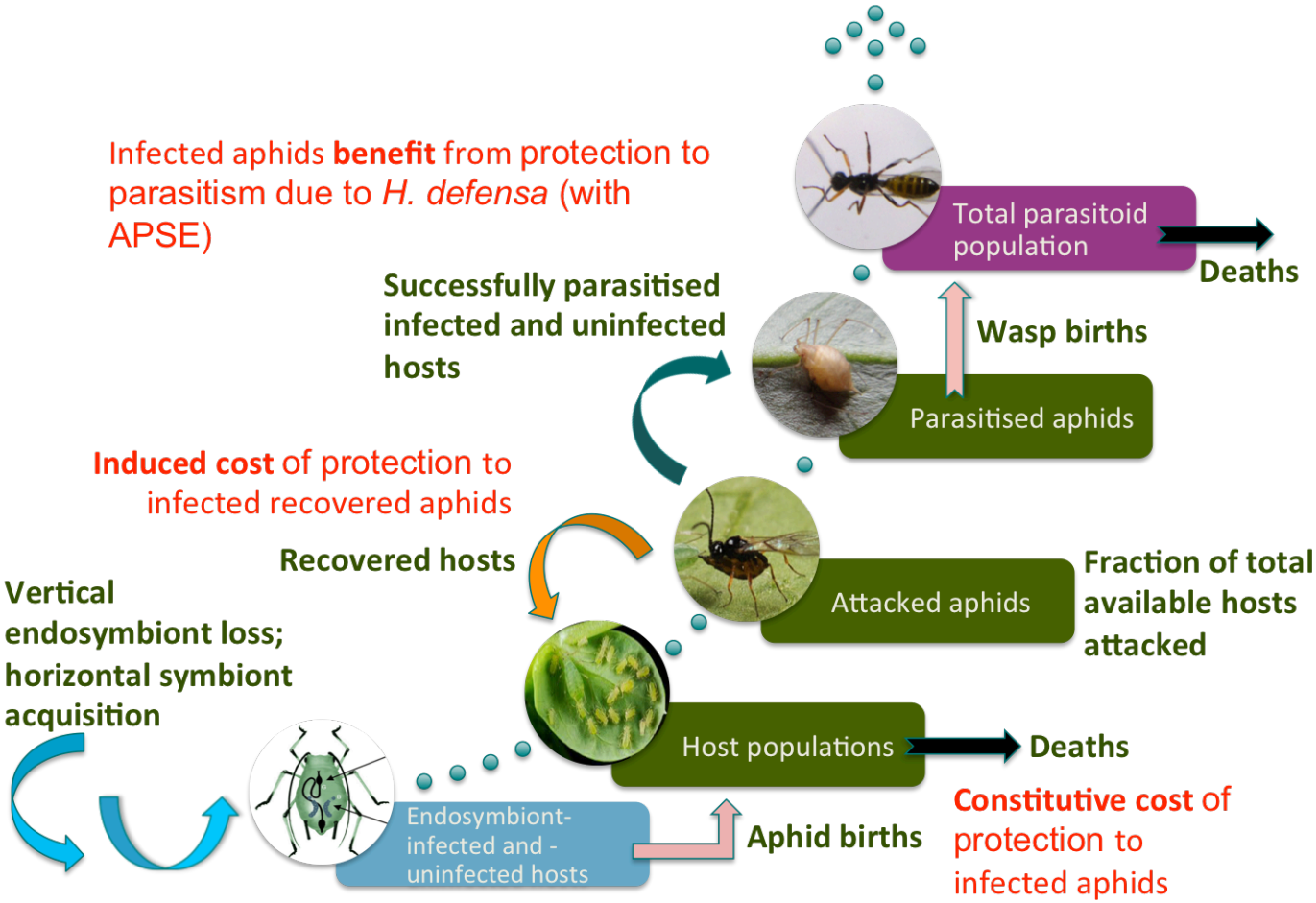
4.1 Introduction

A visual representation of the classes and processes of a mathematical model describing the *A. pisum-A. ervi* biological study system held in culture at JHI and the costs and benefits of harbouring an endosymbiont infection is given in figure 4.1[1,2]. The basic model is based on the general model of Kwiatkowski and Vorburger (2012). All pea aphids harbour the primary endosymbiont *Buchnera* and may harbour a variety of secondary facultative endosymbionts. For the purpose of modelling this study system, "infected" hosts are here classified as harbouring an *H. defensa* endosymbiont infection with associated APSE bacteriophage. Otherwise, for modelling purposes, hosts are considered "uninfected". The term "parasitised hosts" refers in this model to aphids that will definitely be killed by parasitism by a parasitoid wasp. "Attacked hosts" may survive an oviposition attempt and, in this context, survival of an attacked uninfected aphid is considered due to an innate resistance to parasitism and survival of an infected host considered due to the additive effect of innate resistance, together with the resistance conferred by facultative secondary endosymbiont infection. The mechanism by which protection is conferred, for example encapsulation, is not explicitly modelled in this study. Oliver et al. (2012) use the term "parasitism event" to describe an encounter between pea aphid and parasitoid wasp when apparently oviposition takes place; the status of the pea aphid after such an encounter is that of an "attacked host" in the model. Further, they report, that following dissection of attacked pea aphids, usually one parasitoid wasp egg is deposited during each encounter.

[1]Adapted host image reprinted from ISJ, 10, Mandrioli and Manicardi, Evolving aphids: one genome-one organism insects or holobionts?, 1-6, Copyright (2013), with permission M Mandrioli.

[2]Oviposition image courtesy of David Riley at The James Hutton Institute.

Figure 4.1: A visual representation of the classes and processes of the mathematical model describing the *A. pisum*-*A. ervi* biological system.



4.2 Modelling approach

A discrete-time mathematical model of the temporal dynamics of an endosymbiont-host-parasitoid system was developed, extending the work of Kwiatkowski and Vorburger (2012). After exploring their model as presented in their study, using their parameter values arising from a variety of aphid-wasp host-parasitoid systems (table 4.2), a set of parameters was collated for the *A. pisum-A. ervi* biological study system informed as far as possible from experimental work at JHI.

Kwiatkowski and Vorburger (2012) used a system of six coupled difference equations to model the interaction between aphids and parasitoid wasps. In their model, hosts were either infected with facultative endosymbionts or were endosymbiont free. Both host subpopulations were attacked by parasitoid wasps giving rise to subpopulations of infected and uninfected parasitised hosts. A further division was made between infected hosts that had never been attacked and infected hosts that had been attacked but survived. The model equations show how the population sizes change each day. The model runs for a number of days. When considering long-term dynamics, the initial transient dynamics are disregarded when appropriate and simulation results are interpreted according to biologically realistic conditions for population persistence and extinction.

Godfray and Hassell (1991) explored the effect of encapsulation on host-parasitoid population dynamics. After presenting the effects of "all-or-none" encapsulation and "dosage-dependent' encapsulation on the dynamics of the basic Nicholson Bailey model incorporating encapsulation as discussed in section 1.5.5, Godfray and Hassell (1991) go on to consider evolution of the encapsulation response. Their work was found after initial investigation of the Kwiatkowski and Vorburger (2012) model had been carried out and is related to and also used to inform the modelling work in this study.

4.3 Initial model coding

To provide context for the changes made to the Kwiatkowski and Vorburger (2012) model in this study, the authors' original model (section 4.3.1) and the process through which the authors' work was analysed will be presented next. Kwiatkowski and Vorburger (2012) provided code written in Python in their supplemental material to run their model and to reproduce various figures and analysis contained

within. To run the authors' code in the interactive Python console iPython (Pérez and Granger, 2007) as suggested, Anaconda (Continuum-Analytics, 2014) was downloaded and installed. Initially the model was investigated using the authors' code and the figures from their paper reproduced. Then, as part of this study, the model was coded in MATLAB (MATLAB, 2010). The results of simulations using the MATLAB code were checked against output from the Kwiatkowski and Vorburger (2012) code and the figures from their paper were again successfully replicated.

Some of the models written by Godfray and Hassell (1991) were also coded in MATLAB as part of this study and the results of simulations using the MATLAB code checked against figures from their paper and the output of simulations using Populus (Alstad, 2014).

4.3.1 Kwiatkowski and Vorburger (2012) Model

The Kwiatkowski and Vorburger (2012) model parameters are:

b_h birth rate of hosts,

b_p birth rate of parasites,

d_h death rate of hosts,

d_p death rate of parasites,

t_v reliability of vertical transmission of symbionts,

t_h rate of horizontal transmission of symbionts,

p_i innate resistance to parasitism,

p_s symbiont-conferred resistance to parasitism,

c_c constitutive cost of symbiont protection,

c_i induced cost of symbiont protection,

l_k time to kill a successfully parasitised host,

l_e time to emerge from a dead host,

k carrying capacity of host population.

The Kwiatkowski and Vorburger (2012) model equations are:

$$\begin{aligned}
\Delta H_n^0 &= b_h \gamma_n H_n^0 - d_h H_n^0 \\
&\quad -\rho\left(t_h, H_n^0, H_n^s + H_n^r + V_n^s\right) \\
&\quad -\Pi_n^0 + (1 - t_v)(1 - c_c)\, b_h \gamma_n H_n^s \\
&\quad +(1 - t_v)(1 - c_c)(1 - c_i)\, b_h \gamma_n H_n^r \,, \\
\Delta H_n^s &= t_v (1 - c_c)\, b_h \gamma_n H_n^s - d_h H_n^s \\
&\quad +\rho\left(t_h, H_n^0, H_n^s + H_n^r + V_n^s\right) - \theta_n H_n^s \\
&\quad +t_v (1 - c_c)(1 - c_i)\, b_h \gamma_n H_n^r \,, \\
\Delta H_n^r &= -d_h H_n^r - (1 - p_i)(1 - p_s)\, \theta_n H_n^r \\
&\quad +\left[1 - (1 - p_i)(1 - p_s)\right] \theta_n H_n^s \,, \\
\Delta V_n^0 &= \Pi_n^0 - \Pi_{n-l_k}^0 \,, \\
\Delta V_n^s &= \Pi_n^s - \Pi_{n-l_k}^s \,, \\
\Delta P_n &= \Pi_{n-l_k-l_e}^0 + \Pi_{n-l_k-l_e}^s - d_p P_n \,,
\end{aligned}$$

where the model variables are H^0 (hosts without endosymbionts), H^s (hosts with endosymbionts, never attacked), H^r (hosts with endosymbionts, survived attack), V^0 (parasitised hosts without endosymbionts), V^s (parasitised hosts with endosymbionts) and P (parasites). The model equations also use the auxiliary quantities Λ_n, γ_n, θ_n, Π_n^0 and Π_n^0 described in equations 4.1 to 4.5.

The total of the host populations on day *n*, Λ_n, is:

$$\Lambda_n = H_n^0 + H_n^s + H_n^r + V_n^0 + V_n^s \,. \tag{4.1}$$

The so-called carrying capacity enforcing factor in day *n*, γ_n, is:

$$\gamma_n = \max\left(0.0, 1 - \frac{\Lambda_n}{k}\right) . \tag{4.2}$$

The fraction of available hosts attacked on day *n*, θ_n, is:

$$\theta_n = \frac{\rho\left(b_p, H_n^0 + H_n^s + H_n^r, P_n\right)}{H_n^0 + H_n^s + H_n^r} . \tag{4.3}$$

The symbiont-free hosts parasitised on day *n*, Π_n^0, are:

$$\Pi_n^0 = (1 - p_i)\,\theta_n H_n^0 . \tag{4.4}$$

The symbiont-harbouring hosts parasitised on day *n*, Π_n^s, are:

$$\Pi_n^s = (1 - p_i)\,(1 - p_s)\,\theta_n\,(H_n^s + H_n^r) . \tag{4.5}$$

Note that ρ is defined as:

$$\rho\left(b_p, H, P\right) = \frac{b_p H P}{H + b_p P} ,$$

where b_p is defined as the maximum possible number of attacks per single time unit by a parasite.

4.3.2 Population outcomes in the Kwiatkowski and Vorburger (2012) model

The model class of parasites goes extinct if all hosts are extinct. However, if any host population persists, then the parasite population will, in most cases, also persist and this coexistence between parasites and hosts can be stable or oscillatory. To test mathematically for parasitoid wasp extinction in the model, the long term dynamics were examined (with the first 365 time iterations disregarded) and, as suggested by Kwiatkowski and Vorburger (2012), the ratio $\frac{\text{mean of wasp population}}{1+\text{mean of wasp population}}$ goes to zero when the wasp population goes to zero. When all the host populations go extinct, then the quantity Λ used in the Kwiatkowski and Vorburger (2012) model to represent the sum total of hosts goes to zero and the average fraction of protected hosts (represented by μ) is returned as NaN (Not a Number).

Hence there are two measures for looking at extinction.

Looking further at host parasite coexistence, the parasite population can be tested to see if parasite populations are oscillating or not. If parasite populations are cycling, then coexistence with hosts will be oscillatory; if parasite populations are not cycling, then the coexistence will lead to a stable equilibrium. To test for parasite cycling, the standard deviation of the parasite population relative to the mean is calculated (standard deviation of $\frac{\text{parasite population}}{\text{mean parasite population}}$). Again, the first $365\,\text{d}$ were disregarded. The value of this standard deviation quantity tends to zero when the wasp population is stable but the cut off value of this quantity for stability was determined computationally to be around 0.075 for the $3650\,\text{d}$ time duration used in the simulation. This value seemed higher than expected and will be discussed later. If the parasite population goes extinct, then this quantity is returned as NaN.

Whether the parasites are coexisting with the total host population (Λ, not to be confused with the growth parameter λ described in section 1), with just uninfected hosts (H^0), or with just infected hosts ($H^s + H^r$) can be determined by the value of the average fraction of protected hosts, μ. If $\mu = 0$, then the infected hosts, $H^s + H^r$, are extinct and the parasite population coexists with uninfected hosts, H^0. If $\mu = 1$, then uninfected hosts, H^0, are extinct and the parasite population coexists with infected hosts, $H^s + H^r$. If μ is greater than 0 but less than 1, then uninfected hosts, H^0, and infected hosts, $H^s + H^r$, all coexist with the parasite population. If uninfected and infected hosts (H^0 and $H^s + H^r$ respectively) coexist, then the dominant population can be determined by comparing the mean of the protected and unprotected populations, again ignoring the first $365\,\text{d}$ of the simulation.

The flow chart (4.2) summarises these possibilities and was used to write code to identify different types of population dynamics from the model output.

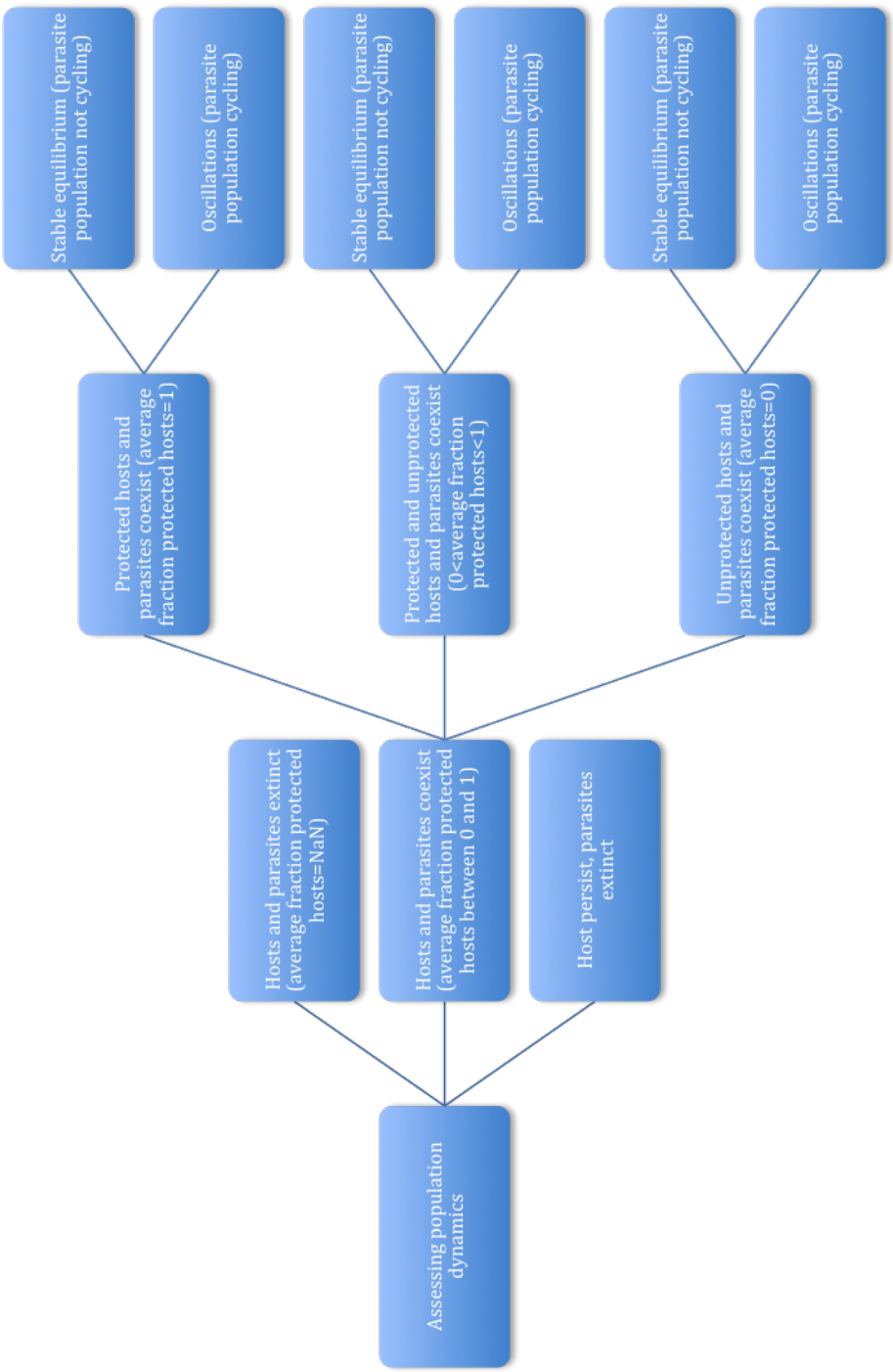


Figure 4.2: Analysis of population dynamics using Kwiatkowski and Vorburger (2012) model code.

4.3.3 Anomalies arising from the Kwiatkowski and Vorburger (2012) model

Initial exploration of the model presented by Kwiatkowski and Vorburger (2012) using their code yielded some unexpected results. Figure 4.3 shows that the resulting dynamics are, in some cases, dependent on the initial host population sizes. Reversing the initial balance between uninfected and infected hosts changed the dynamics when the strength of endosymbiont infection was $p_s = 0.75$ from oscillatory coexistence to protected hosts prevailing. This appeared to be due to the way the authors implemented their mathematical model and then subsequently coded it.

Recall that density dependent growth is included in the Kwiatkowski and Vorburger (2012) model using a density dependent factor, γ_n:

$$\gamma_n = \max\left(0.0, 1 - \frac{\Lambda_n}{k}\right) , \tag{4.6}$$

where Λ_n is the total of the host subpopulations (including parasitised but not yet dead hosts) on day *n* and k is carrying capacity of the host populations. Note that this expression has a minimum value of zero and is also referred to by the authors as the "carrying capacity enforcing factor on day *n*". The inclusion of this term limits any oscillatory approach to equilibrium to a gradual, near step-wise, return to equilibrium for values above the carrying capacity.

The authors also attempt to introduce biological realism into their model by applying the following criteria to subpopulation changes where X_n is each subpopulation (H^0, H^s, H^r, V^0, V^s and P) on day n and ΔX_n is the change in that subpopulation on that day:

$$X_{n+1} = \begin{cases} X_n + \Delta X_n & \text{if } X_n + \Delta X_n \geq 1.0 \ , \\ 0.0 & \text{otherwise} \ . \end{cases} \tag{4.7}$$

If a total subpopulation size accounting for the change in that subpopulation on that day ($X_n + \Delta X_n$) becomes less than 1 individual, then the size of that subpopulation is set to zero on the next day even during the initial period of transient dynamics. The effect of this is, under some circumstances, to force a subpopulation to become extinct before the initial transient period is over and before the

long term population dynamics are established. So long as each total subpopulation size remains greater or equal to 1 individual, then no artificial extinction constraints are applied to the population dynamics.

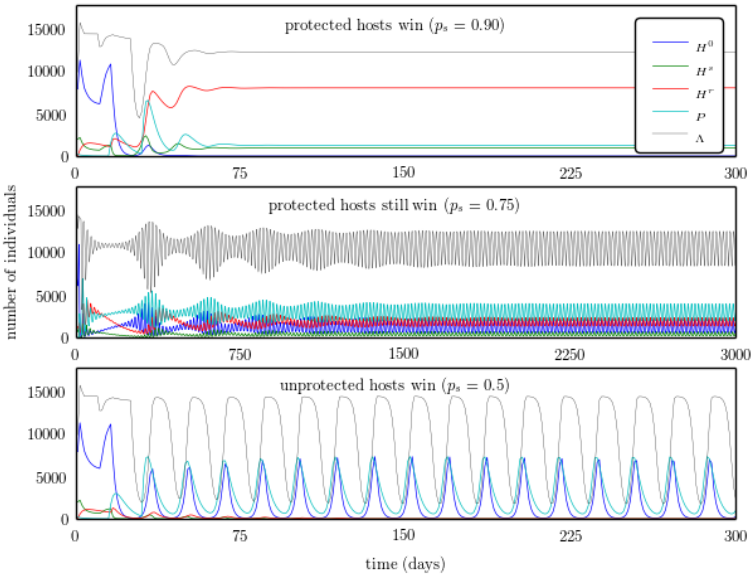
Kwiatkowski and Vorburger (2012) report that the stability of their model cannot be analysed using standard stability analysis because of higher order terms used in their model. Analysis of their model is further complicated by the use of a custom ternary function, ρ, used to model parasitism:

$$\rho\left(b_p, H, P\right) = \frac{b_p H P}{H + b_p P} \ , \tag{4.8}$$

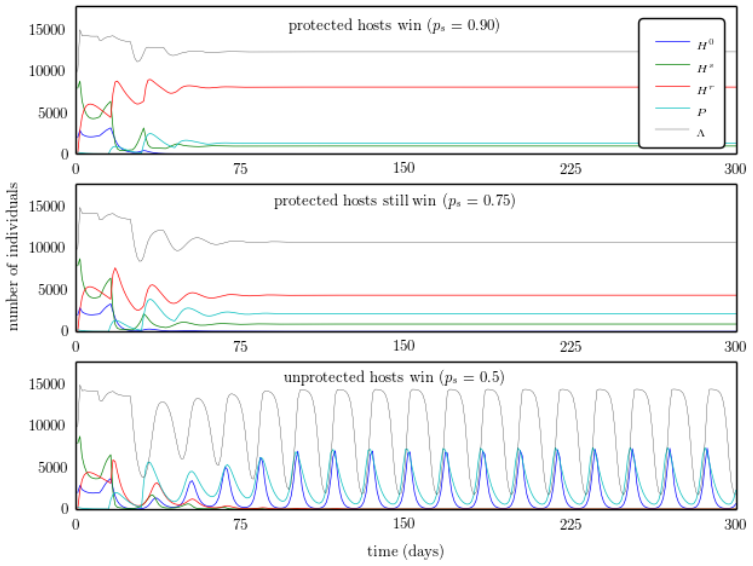
where b_p is defined as the maximum possible number of attacks per single time unit by a parasite, H is the relevant host population(s) and P is the parasite population.

The same construct is used similarly to model the horizontal transmission of endosymbionts between infected and uninfected hosts. In this second use of the custom ternary function, the number of "symbiont transactions" per unit time is obtained from $\rho\left(t_h, H^-, H^+\right)$, where t_h is an endosymbiont "basal infectivity" and $H^-$and H^+ are the host infection-recipient and infection-donor populations respectively.

When coding their model in Python, the authors use a FIFO (First In, First Out) queuing system into which parasitised hosts are allocated for the duration of time equal to the time taken to kill a successfully parasitised host, l_k, and then into a further FIFO queue into which dead hosts are allocated for a time equal to the time for a wasp to then emerge from a dead aphid host, l_e. This lengthens the code needed to implement the model and makes it harder to identify and analyse the time-delays, l_k and l_e, implicit in the model. Again, exploration of the model presented by Kwiatkowski and Vorburger (2012) using their code yielded an unexpected abrupt switch in the type of resulting population dynamics when the time delays were varied only slightly, as shown in Figure 4.4.

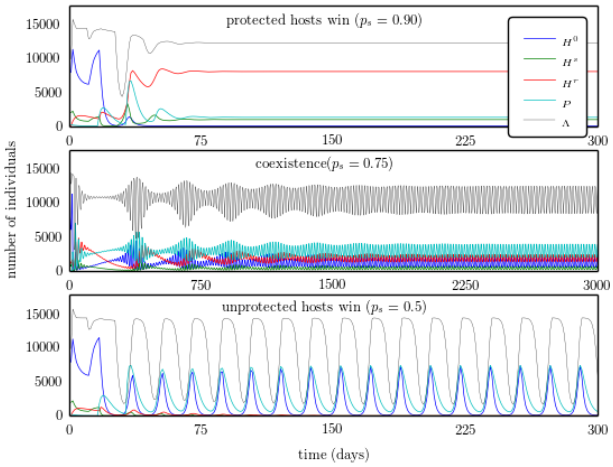


(a) Use of authors' code to replicate their figure 4 with initial conditions H^s=2000, H^0=8000, P=200.

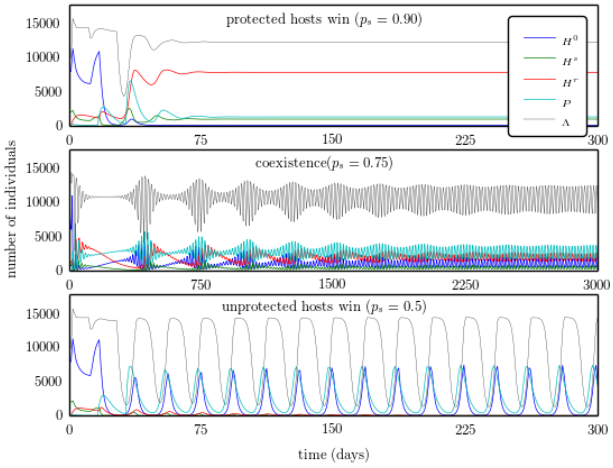


(b) Initial conditions reversed to H^0=2000, H^s=8000, P=200.

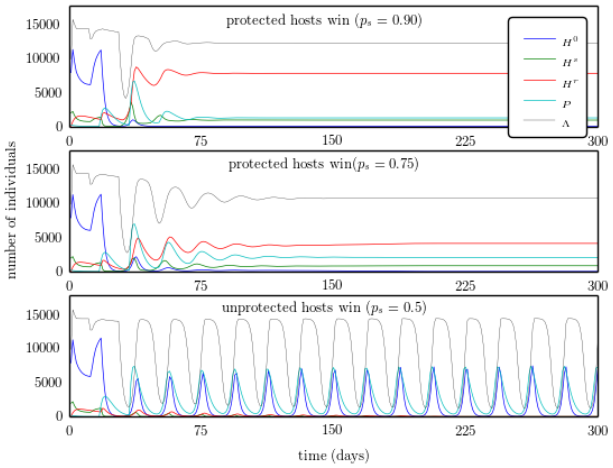
Figure 4.3: Effects on dynamics of reversing initial population sizes using Kwiatkowski and Vorburger (2012) code



(a) Wasps take 1 extra day to emerge



(b) Hosts take 1 extra day to die



(c) Extra day to die, extra day to emerge

Figure 4.4: Varying wasp parameters (implicit time delays)

4.4 The revised model

Following exploration of the Kwiatkowski and Vorburger (2012) model using the authors' code, the first part of this study involved coding an identical version of the model in MATLAB using the same FIFO queue structure. This code was used to replicate the figures from the original paper. The same results for both the simulations contained within the paper and the simulations done to explore the model were obtained. It was then decided to simplify the code where possible and to refine the mathematical expression of the model to remove the limitations placed on state variables described in section 4.3.3 and to enable some attempt at more formal stability analysis using MAPLE^{TM}. This was done in a gradual manner, introducing just one change at a time and checking that the code gave sensible results when initial simulations were run at each stage.

The final model presented here is based on the same interactions between hosts and parasitoids proposed by Kwiatkowski and Vorburger. The key differences are:

- replacement of the authors' custom ternary functions to model parasitism and horizontal transmission of endosymbionts,
- modification to how density dependent growth in the host population is expressed,
- removal of the authors' requirements that subpopulation sizes are set to zero if they go below one individual during a simulation,
- introduction of a type II functional response typical for such parasitism,
- allowance for change in the strength of protection of the symbiont when an aphid is attacked for a second (or subsequent) time as suggested by experimental evidence.

4.4.1 State variables

The model variables are the population sizes of hosts without endosymbionts, H_0, hosts with endosymbionts that have never been attacked, H_S, hosts with endosymbionts that have survived attack, H_R, parasitised hosts without endosymbionts, V_0, parasitised hosts with endosymbionts, V_S, and parasitoids, P, i.e..

H_0 Hosts without endosymbionts,

H_S Hosts with endosymbionts (never attacked),

H_R Hosts with endosymbionts (survived attack),

V_0 Parasitised hosts without endosymbionts,

V_S Parasitised hosts with endosymbionts,

P Parasitoids.

4.4.2 Model parameters

Endosymbiont acquisition is a horizontal process; endosymbiont loss is a vertical process. Horizontal transmission of endosymbionts is from infected hosts (both unparasitised and parasitised) to uninfected unparasitised hosts at a rate t_h. Host reproduction is parthenogenic with the fidelity of vertical maternal transmission of endosymbionts t_v. All host subpopulations are modelled (in the absence of parasitism) as having the same density dependent birth rate, b_h, and density independent death rate, d_h. The total host population has carrying capacity k. Retaining a density independent death rate is a limitation imposed by the lack of an explicit density dependent pea aphid death rate in either previous experimental work or the published literature. The inclusion of a density dependent birth rate informed by experimental data allows broad comparison between this model and the density dependent modelling work of Godfray and Hassell (1991) and removes the artificial constraints on oscillatory approaches to equilibrium in the Kwiatkowski and Vorburger (2012) model. The expression for the density dependent birth rate arises from modification to a basic density independent discrete difference equation for population growth:

$$H_{t+1} = H_t + b_h H_t - d_h H_t \ , \tag{4.9}$$

where H_t is the host population on day t, b_h is the birth rate and d_h is the death rate. The density dependent birth rate is given by:

$$b_h - a\lambda_t \ , \tag{4.10}$$

where a is the amount by which the per capita birth rate changes as a result of the addition of a further individual to the total host population. This yields a partially

density dependent model:

$$H_{t+1} = H_t + (b_h - a\lambda_t)\, H_t - d_h H_t \ . \tag{4.11}$$

Since, at equilibrium, $H_t = H_{t+1} = H_{eq}$, and, further, $\lambda_{eq} = k$ then:

$$H_{eq} = H_{eq} + (b_h - a\lambda_{eq})\, H_{eq} - d_h H_{eq} \ . \tag{4.12}$$

So

$$a = \frac{b_h - d_h}{k} \ . \tag{4.13}$$

Substituting for a gives

$$H_{t+1} = H_t + \left(b_h - \frac{(b_h - d_h)\, \lambda_t}{k}\right) H_t - d_h H_t \ . \tag{4.14}$$

Parasitism here is based on the approach taken by the Nicholson-Bailey model (Nicholson and Bailey, 1935) that assumes that the probability of host being not found follows a Poisson distribution and modelled by a type II functional response with parasitoid search efficiency, a, that determines host escape and the number of hosts parasitised and handling time, b. If the handling time, b is zero, the functional response reverts to a basic linear type I functional response. Ives et al. (1999) suggest that many factors, including host density and host stage of development, contribute to the variability of *A. ervi* foraging efficiency on pea aphids both between and within host plants. The net effect reduces the strength of a type II functional response to a near type I response in this host-parasitoid system. He et al. (2006) investigate the relationship between host density and reproductive fitness (parasitoid reproductive output and sex allocation) of *A. ervi*. They provide evidence that *A. ervi* responds to increasing *A. pisum* host density so altering oviposition strategy by increasing the rate at which hosts are parasitised and by decreasing the effort put into superparasitism. The change in parasitism rate in the experiment carried out by He et al. (2006) is suggestive of a classic type II functional response. The horizontal transmission of endosymbionts is similarly modelled following a Poisson distribution (Lively et al., 2005). The parameters describing the costs and benefits of endosymbiont infection are described in the context of the biological study system in figure 4.1. The time delays in the model are l_k and l_e, the times to kill and emerge from hosts respectively.

The following is a summary of the quantities that appear in this analysis:

b_h Host birth rate,

d_h Host death rate,

d_p Parasitoid death rate,

t_v Reliability of vertical transmission,

t_h Rate of horizontal symbiont acquisition,

p_i Innate resistance to parasitism,

p_s Resistance to parasitism provided by endosymbiont (first attack),

p_{sp} Resistance to parasitism provided by endosymbiont (second and subsequent attacks),

c_c Constitutive cost of endosymbiont infection,

c_i Induced cost of endosymbiont infection,

l_k Time for developing parasitoid to kill a parasitised host,

l_e Time for parasitoid to emerge from dead host,

k Carrying capacity of host population,

a Parasitoid search efficiency (for type II functional response),

b Handling time (for type II functional response).

4.4.3 Model explanation and assumptions

In a discrete time population model:

$$\#\text{in future} = \#\text{now} + \#\text{born} - \#\text{died} + \#\text{immigrated} - \#\text{emigrated} \,. \quad (4.15)$$

Table 4.1 describes how the population changes in this model each day as described by equation 4.15.

Table 4.1: Population changes each day in the model

Population change:	Born or immigrated (+ve)	Died or emigrated (-ve)
H_0	offspring born to H_0	H_0 that died
	offspring born to H_S that lost symbiont vertically	H_0 that acquired symbiont horizontally
	offspring born to H_R that lost symbiont vertically	H_0 attacked and parasitised
H_S	offspring born to H_S that kept symbiont	H_S that died
	offspring born to H_R that kept symbiont	all H_S attacked
	H_0 that acquired symbiont horizontally	
H_R	H_S attacked but survived	H_R that died
		H_R attacked and parasitised
V_0	H_0 that were parasitised	parasitised H_0 now killed by parasitism
V_S	H_S attacked and parasitised	parasitised H_S now killed by parasitism
	H_R attacked and parasitised	parasitised H_R now killed by parasitism
P	wasps emerging from dead parasitised H_0	wasps that died
	wasps emerging from dead parasitised H_S aphids	
	wasps emerging from dead parasitised H_R aphids	

The model assumptions are:

- unparasitised hosts reproduce at constant rate throughout their life but parasitised host do not reproduce,
- the first time that symbiont protected hosts are attacked they have protection p_s due to the symbiont; if survived and attacked a second or subsequent time the protection is p_{sp},
- one wasp egg is deposited each time an aphid is attacked,
- parasitised hosts (those that will definitely be killed by parasitism after attack) are not attacked again,
- all hosts have some innate resistance to parasitism, assumed to be the same,
- apart from a reproductive cost to their hosts, endosymbionts do not otherwise affect their hosts, the parasitoids or any host-parasitoid interaction,
- parasitoids do not distinguish between infected and uninfected hosts; encounters with hosts are random.

4.4.4 Parameter values

Initial numerical simulations use generic aphid-wasp parameter values from Kwiatkowski and Vorburger (2012) with the exception of parasitoid search efficiency and handling time data informed by Snyder and Ives (2003).

A secondary set of parameters specific to pea aphids was gathered from previous experimental work and the published literature as follows.

The effective aphid fecundity is given by the number of offspring produced in a time equal to the pre-reproductive time (time to reproduction). Using data collected during a previous fitness assay (figure 4.5) using pea aphids from secondary endosymbiont-free clonal line LL01 gave a mean rate of nymph production per aphid of 4.9 ± 0.4 d^{-1} (Cornwell, 2011) at $20\,$°C. In the published literature, Morgan et al. (2001) report results from a fitness experiment carried out using a very similar method rearing pea aphids on two pea cultivars (Sancho and Scout) at a range of different temperatures and report that the mean numbers of nymphs per adult per day at $19.6\,$°C were 5.5 ± 0.3 d^{-1} and 4.6 ± 0.5 d^{-1} respectively. The secondary endosymbiont infection status of their culture maintained from pea

Figure 4.5: Reproductive output from adult pea aphids from clonal line LL01 during prior fitness assay

aphids collected in the east of England, UK, is unknown as the experimental work pre-dates identification and routine screening for infection status. However, data noted incidentally on another pea aphid line free from protective secondary endosymbiont infection (JF200) during the course of nymph generation for assays to look at superparasitism, appeared to suggest that this line had a lower mean rate of nymph production. This may be due to genotypic differences between clonal lines. Laughton et al. (2014) report that the cumulative offspring of a secondary endosymbiont line was approximately 100 nymphs over a period of just over $35\,\mathrm{d}$ yielding a mean birth rate of approximately $2.8\,\mathrm{d}^{-1}$. The initial rate of nymph production was approximately $4\,\mathrm{d}^{-1}$. Hence, a biologically reasonable estimate for the host lifetime birth rate is taken to be $\leq 5\,\mathrm{d}^{-1}$.

Kwiatkowski and Vorburger (2012) take the mean host life span to be $20\,\mathrm{d}$ and mean parasitoid life span to be $3.5\,\mathrm{d}$ and use the inverse of these as the host and parasitoid death rates respectively, noting that the results of their simulations were robust to small changes in these parameters. Morgan et al. (2001) note considerable variation in measured median life spans when comparing their results to those in the published literature. Taking this into account, it is reasonable to simulate host lifespans in excess of $20\,\mathrm{d}$ but not exceeding $40\,\mathrm{d}$. Bai (1991) investigated life history and reproductive strategies in parasitoid wasps including *A. ervi* and

reports peak egg load in *A. ervi* at 4 d to 6 d after emergence from the aphid host. Comparing *A. ervi* with *Aphelinus asychis*, another parasitoid wasp emerging with a low mature egg load, it was noted that the *A. ervi* egg load was consistently an order of magnitude higher than in *A. asychis* during the first 10 d post-emergence. This, in addition to observed oviposition strategies, suggests that *A. ervi* parasitism is time, not egg, limited. Parasitism assays carried out in this study used parasitoid wasps aged between 2 d to 5 d. It is reasonable to simulate the parasitoid lifespan as in excess of 5 d but not exceeding 14 d.

The resistance to parasitism of clonal line LL01 was also taken to be a measure of the innate resistance to parasitism of a secondary endosymbiont free pea aphid. Data collected during two experiments to measure resistance of LL01 nymphs to *A. ervi* parasitoid wasps gave a fractional mean innate resistances of 0.55 ± 0.41 and 0.63 ± 0.35 (Cornwell, 2011). The former innate resistance was measured using offspring of the nymphs from whom the birth rate was calculated in an experiment using a foraging parasitoid wasp on a caged bean plant and the latter innate resistance was measured using nymphs reared concurrently in the stock aphid cultures using a foraging wasp in a petri dish experimental arena. Data collected for clonal line LL01 used in the preliminary assays to assess the susceptibility of pea aphids harbouring PAXS to parasitism (section 2.4.3.1) yielded a mean innate resistance to parasitism of 0.51 ± 0.25. Given the large standard deviation in the results, it is not unreasonable to take, as an initial parameter value, the fractional innate resistance to parasitism to be 0.55.

Data collected during the same previous fitness assay with pea aphids from *H. defensa* with APSE infected clonal lines JF01S and JF99/04 (previously held at JHI) gave a mean rate of nymph production across both lines of 2.2 ± 1.1 d^{-1} (Cornwell, 2011). Given that a constitutive cost of 1 corresponds to full reproduction sterilisation, a decrease in nymph generation from ≈ 5 d^{-1} to $2.2\,\text{d}^{-1}$ would be caused by a reduction by a factor of ≈ 0.45 and hence a constitutive cost of ≈ 0.55.

The resistance to parasitism of clonal lines JF01S and JF99/04 was used to estimate the protection conferred to pea aphids by an *H. defensa* endosymbiont infection. Data collected during two experiments to measure resistance of these nymphs to *A. ervi* parasitoid wasps gave a mean fractional total resistances of 0.62 ± 0.33 and 0.70 ± 0.26 (Cornwell, 2011). The former mean fractional total resistance was measured using offspring of the nymphs from whom the birth rate

was calculated in an experiment using a foraging parasitoid wasp on a caged bean plant and the latter innate resistance was measured using nymphs reared concurrently in the stock aphid cultures using a foraging wasp in a petri dish experimental arena. Data collected for clonal line N117 used in the preliminary assays to assess the susceptibility of pea aphids harbouring PAXS to parasitism (section 2.4.3.1) yielded a mean fractional total resistance to parasitism of 0.66 ± 0.34. When discussing experimental results from parasitism assays of singly versus double parasitised aphids, Oliver et al. (2012) make reference to the significant variation of protection afforded to pea aphids by difference strains of *H. defensa* carrying different strains of APSE bacteriophage. No molecular characterisation data is available about the specific *H. defensa* strains present in these clonal lines. The overall range from experimental data for innate resistance to parasitism is $0.14 \leq p_i \leq 0.98$ and the overall range from experimental data for total resistance to parasitism is $0.29 \leq p_i + p_s \leq 1.0$. Hence, there is a wide overlap of values.

Bai (1991) report that the development time from egg to emergent adult wasp is temperature dependent and typically takes two weeks at $21\,^{\circ}$C hence $l_k + l_e = 14$ remains a realistic parameter estimate for this system. Again, the parasitoid search efficiency and handling time parameters are taken from experimental assays by Snyder and Ives (2003).

The rate of horizontal symbiont acquisition, t_h, was taken to be less than 1×10^{-3} and the fidelity of vertical transmission as no lower than 0.8 in laboratory conditions (A. Karley, *pers. comm.*, 2013). This is a narrower range of transmission values than used by Kwiatkowski and Vorburger (2012) but is based on data and observation of the *A. pisum*-*A. ervi* study system. In their study, the authors carry out simulations holding one transmission rate constant whilst varying the other rather than exploring the effects of varying both parameters at the same time.

In summary:

$b_h \leq 5$ Host birth rate (d^{-1}),

$\frac{1}{40} \leq d_h \leq \frac{1}{20}$ Host death rate (d^{-1}),

$\frac{1}{14} \leq d_p \leq \frac{1}{5}$ Parasitoid death rate (d^{-1}),

$0.8 \leq t_v \leq 1.0$ Reliability of vertical transmission,

$0.0 \leq t_h \leq 0.001$ Rate of horizontal symbiont acquisition,

$p_i = 0.55$ Innate resistance to parasitism,

$0 \leq p_s \leq 1$ Resistance to parasitism provided by endosymbiont (first attack),

$0 \leq p_{sp} \leq xp_s$ Resistance to parasitism provided by endosymbiont (second and subsequent attacks) where x is the factor determining the change in endosymbiont mediated protection,

$0 \leq c_c \leq 1$ Constitutive cost of endosymbiont infection,

$0 \leq c_i \leq 1$ Induced cost of endosymbiont infection,

$l_k = 9$ Time for developing parasitoid to kill a parasitised host (d),

$l_e = 5$ Time for parasitoid to emerge from dead host (d),

$l_k + l_e = 14$ Total time from oviposition to emergence (d),

$k = 15000$ Carrying capacity of host population (individuals),

$a = 146$ Parasitoid search efficiency (for Type II functional response),

$b = 0.0011$ Handling time (for Type II functional response).

The Kwiatkowski and Vorburger (2012) default set of parameter values and the pea aphid specific set of parameters are compared in table 4.2.

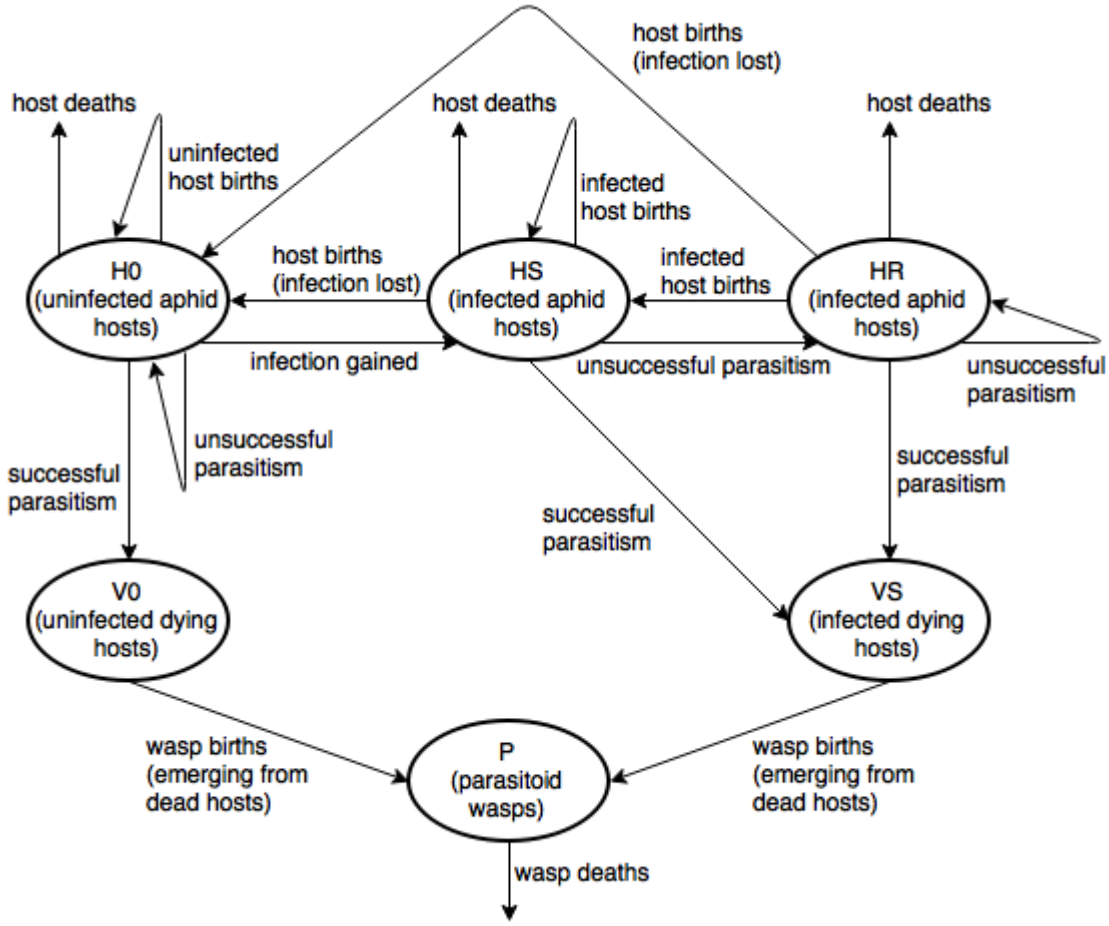
4.4.5 Model equations

The interactions between model classes described by the model equations are shown in figure 4.6.

Table 4.2: Comparison of the general aphid parameter values termed default in the Kwiatkowski and Vorburger (2012) study and the pea aphid parameter values used in this study.

Values from general aphid data	**Values from pea aphid data**
b_h =2 d^{-1}	$b_h \leq$5 d^{-1}
b_p =30 d^{-1}	-
d_h =0.05 d^{-1}	$\frac{1}{40}$ $\mathrm{d}^{-1} \leq d_h \leq \frac{1}{20}$ d^{-1}
d_p =0.286 d^{-1}	$\frac{1}{14}$ $\mathrm{d}^{-1} \leq d_p \leq \frac{1}{5}$ d^{-1}
$t_v = 0.995$	$0.8 \leq t_v \leq 1.0$
$t_h = 0.001$	$0.0 \leq t_h \leq 0.001$
$p_i = 0.5$	$p_i = 0.55$
$p_s = 0.9$	$0 \leq p_s \leq 1$
-	$0 \leq p_{sp} \leq xp_s$
$c_c = 0.0$	$0 \leq c_c \leq 1$
$c_i = 0.0$	$0 \leq c_i \leq 1$
l_k =9 d	l_k =9 d
l_e =5 d	l_e =5 d
$l_k + l_e$ =14 d	$l_k + l_e$ =14 d
$k = 15000$ individuals	$k = 15000$ individuals
-	$a = 146$
-	$b = 0.0011$

Figure 4.6: Interactions between model classes described by model equations.



The complete mathematical model is written in full here; the MATLAB code (Appendix A) uses auxiliary quantities for total hosts, fraction of available hosts attacked, successful attacks on uninfected and infected hosts and horizontal acquisition of endosymbionts to simplify implementation of the model.

The system of difference equations is thus as follows:

$$
\begin{aligned}
H_0(t+1) = H_0(t) + \\
&\left[b_h - \frac{(b_h - d_h)(H_0(t) + H_S(t) + H_R(t) + V_0(t) + V_S(t))}{k}\right] H_0(t) - \\
&d_h H_0(t) - \left[1 - \exp\left\{-\frac{t_h(H_S(t) + H_R(t) + V_S(t))}{H_0(t) + H_S(t) + H_R(t) + V_0(t) + V_S(t)}\right\}\right] H_0(t) - \\
&(1 - p_i)\left[1 - \exp\left\{\frac{-aP(t)}{(1 + ab(H_0(t) + H_S(t) + H_R(t)))}\right\}\right] H_0(t) + \\
&(1 - t_v)(1 - c_c)\left[b_h - \frac{(b_h - d_h)(H_0(t) + H_S(t) + H_R(t) + V_0(t) + V_S(t))}{k}\right] H_S(t) + \\
&(1 - t_v)(1 - c_c)(1 - c_i)\left[b_h - \frac{(b_h - d_h)(H_0(t) + H_S(t) + H_R(t) + V_0(t) + V_S(t))}{k}\right] H_R(t) \ ,
\end{aligned}
$$

$$
\begin{aligned}
H_S(t+1) = H_S(t) + \\
& t_v(1-c_c)\left[b_h - \frac{(b_h-d_h)(H_0(t)+H_S(t)+H_R(t)+V_0(t)+V_S(t))}{k}\right] H_S(t) - \\
& d_h H_S(t) + \left[1 - \exp\left\{-\frac{t_h(H_S(t)+H_R(t)+V_S(t))}{H_0(t)+H_S(t)+H_R(t)+V_0(t)+V_S(t)}\right\}\right] H_0(t) - \\
& \left[1 - \exp\left\{\frac{-aP(t)}{(1+ab(H_0(t)+H_S(t)+H_R(t)))}\right\}\right] H_S(t) + \\
& t_v(1-c_c)(1-c_i)\left[b_h - \frac{(b_h-d_h)(H_0(t)+H_S(t)+H_R(t)+V_0(t)+V_S(t))}{k}\right] H_R(t)\ ,
\end{aligned}
$$

$$
\begin{aligned}
H_R(t+1) = H_R(t) - \\
& d_h H_R(t) - (1-p_i)(1-p_{sp})\left[1 - \exp\left\{\frac{-aP(t)}{(1+ab(H_0(t)+H_S(t)+H_R(t)))}\right\}\right] H_R(t) + \\
& [1-(1-p_i)(1-p_s)]\left[1 - \exp\left\{\frac{-aP(t)}{(1+ab(H_0(t)+H_S(t)+H_R(t)))}\right\}\right] H_S(t)\ ,
\end{aligned}
$$

$$V_0(t+1) = V_0(t) +$$

$$(1-p_i)\left[1-\exp\left\{\frac{-aP(t)}{(1+ab(H_0(t)+H_S(t)+H_R(t)))}\right\}\right]H_0(t) -$$

$$(1-p_i)\left[1-\exp\left\{\frac{-aP(t-l_k)}{(1+ab(H_0(t-l_k)+H_S(t-l_k)+H_R(t-l_k)))}\right\}\right]H_0(t-l_k)\ ,$$

$$V_S(t+1) = V_S(t) +$$

$$(1-p_i)(1-p_s)\left[1-\exp\left\{\frac{-aP(t)}{(1+ab(H_0(t)+H_S(t)+H_R(t)))}\right\}\right]H_S(t) +$$

$$(1-p_i)(1-p_{sp})\left[1-\exp\left\{\frac{-aP(t)}{(1+ab(H_0(t)+H_S(t)+H_R(t)))}\right\}\right]H_R(t) -$$

$$(1-p_i)(1-p_s)\left[1-\exp\left\{\frac{-aP(t-l_k)}{(1+ab(H_0(t-l_k)+H_S(t-l_k)+H_R(t-l_k)))}\right\}\right]H_S(t-l_k) -$$

$$(1-p_i)(1-p_{sp})\left[1-\exp\left\{\frac{-aP(t-l_k)}{(1+ab(H_0(t-l_k)+H_S(t-l_k)+H_R(t-l_k)))}\right\}\right]H_R(t-l_k)\ ,$$

$$
\begin{aligned}
P(t+1) = P(t) + \\
&(1-p_i)\left[1-\exp\left\{\frac{-aP(t-l_k-l_e)}{(1+ab(H_0(t-l_k-l_e)+H_S(t-l_k-l_e)+H_R(t-l_k-l_e)))}\right\}\right] H_0(t-l_k-l_e) + \\
&(1-p_i)(1-p_s)\left[1-\exp\left\{\frac{-aP(t-l_k-l_e)}{(1+ab(H_0(t-l_k-l_e)+H_S(t-l_k-l_e)+H_R(t-l_k-l_e)))}\right\}\right] H_S(t-l_k-l_e) + \\
&(1-p_i)(1-p_{sp})\left[1-\exp\left\{\frac{-aP(t-l_k-l_e)}{(1+ab(H_0(t-l_k-l_e)+H_S(t-l_k-l_e)+H_R(t-l_k-l_e)))}\right\}\right] H_R(t-l_k-l_e) - \\
&d_pP(t)\ .
\end{aligned}
$$

4.4.6 Equilibria

At stable equilibrium (indicated by *):

$$
\begin{aligned}
H_0^* = H_0^* + & \\
& \left[b_h - \frac{(b_h - d_h)(H_0^* + H_S^* + H_R^* + V_0^* + V_S^*)}{k} \right] H_0^* - \\
& d_h H_0^* - \left[1 - \exp\left\{ - \frac{t_h (H_S^* + H_R^* + V_S^*)}{H_0^* + H_S^* + H_R^* + V_0^* + V_S^*} \right\} \right] H_0^* - \\
& (1 - p_i) \left[1 - \exp\left\{ \frac{-aP^*}{(1 + ab(H_0^* + H_S^* + H_R^*))} \right\} \right] H_0^* + \\
& (1 - t_v)(1 - c_c) \left[b_h - \frac{(b_h - d_h)(H_0^* + H_S^* + H_R^* + V_0^* + V_S^*)}{k} \right] H_S^* + \\
& (1 - t_v)(1 - c_c)(1 - c_i) \left[b_h - \frac{(b_h - d_h)(H_0^* + H_S^* + H_R^* + V_0^* + V_S^*)}{k} \right] H_R^* \, ,
\end{aligned}
$$

$$
\begin{aligned}
H_S^* = H_S^* + & \\
& t_v (1 - c_c) \left[b_h - \frac{(b_h - d_h)(H_0^* + H_S^* + H_R^* + V_0^* + V_S^*)}{k} \right] H_S^* - \\
& d_h H_S^* + \left[1 - \exp\left\{ - \frac{t_h (H_S^* + H_R^* + V_S^*)}{H_0^* + H_S^* + H_R^* + V_0^* + V_S^*} \right\} \right] H_0^* - \\
& \left[1 - \exp\left\{ \frac{-aP^*}{(1 + ab(H_0^* + H_S^* + H_R^*))} \right\} \right] H_S^* + \\
& t_v (1 - c_c)(1 - c_i) \left[b_h - \frac{(b_h - d_h)(H_0^* + H_S^* + H_R^* + V_0^* + V_S^*)}{k} \right] H_R^* \, ,
\end{aligned}
$$

$$H_R^* = H_R^* -$$

$$d_h H_R^* - (1-p_i)(1-p_{sp})\left[1-\exp\left\{\frac{-aP^*}{(1+ab(H_0^*+H_S^*+H_R^*))}\right\}\right]H_R^* +$$

$$[1-(1-p_i)(1-p_s)]\left[1-\exp\left\{\frac{-aP^*}{(1+ab(H_0^*+H_S^*+H_R^*))}\right\}\right]H_S^* ,$$

$$V_0^* = l_k(1-p_i)\left[1-\exp\left\{\frac{-aP^*}{(1+ab(H_0^*+H_S^*+H_R^*))}\right\}\right]H_0^*$$

$$V_S^* =$$

$$l_k(1-p_i)(1-p_s)\left[1-\exp\left\{\frac{-aP^*}{(1+ab(H_0^*+H_S^*+H_R^*))}\right\}\right]H_S^* +$$

$$l_k(1-p_i)(1-p_{sp})\left[1-\exp\left\{\frac{-aP^*}{(1+ab(H_0^*+H_S^*+H_R^*))}\right\}\right]H_R^* ,$$

$$P^* = P^* +$$

$$(1-p_i)\left[1-\exp\left\{\frac{-aP^*}{(1+ab(H_0^*+H_S^*+H_R^*))}\right\}\right]H_0^* +$$

$$(1-p_i)(1-p_s)\left[1-\exp\left\{\frac{-aP^*}{(1+ab(H_0^*+H_S^*+H_R^*))}\right\}\right]H_S^* +$$

$$(1-p_i)(1-p_{sp})\left[1-\exp\left\{\frac{-aP^*}{(1+ab(H_0^*+H_S^*+H_R^*))}\right\}\right]H_R^* -$$

$$d_p P^* .$$

4.4.7 Coding and interpreting population outcomes

As there is little biological sense, at this point, in considering the output of the H_S and H_R populations individually given the model assumption that parasitoids

do not discriminate between infected and uninfected hosts and given that there is no corresponding subdivision in the model for uninfected hosts, the total infected host population $H_S + H_R$ is calculated as an output from the model and used in the interpretation of the results from numerical simulations.

The long term host-parasitoid dynamics of the system were interpreted for each simulation. Firstly, the long term stability was assessed by looking at the oscillatory nature, if any, of the parasitoid population. Considering the last $100\,\mathrm{d}$ of the simulation only, if the standard deviation of the wasp population relative to the mean wasp population gets closer to zero, then the population dynamics become more stable. Through numerical simulation, the threshold for population stability in the revised model was taken to be:

$$\text{std}\left[\frac{P}{\text{mean}(P)}\right] < 0.0001\ . \tag{4.16}$$

This was a much smaller value for the standard deviation compared to the value of 0.075 computed as the threshold for population stability in the Kwiatkowski and Vorburger (2012) model as originally coded (section 4.3.2). MAPLE was used to verify the size of the equilibrium populations for some of the stable population simulation outcomes.

Biologically realistic population extinction was assessed by looking to see if the host populations dropped below 1 individual over the final 100 days of the simulation once final dynamics were established, rather than during the course of the simulation. The final population values were set as:

$$\text{Final } H_0 = \begin{cases} H_0 & \text{if } \min H_0 \geq 1\ , \\ 0 & \text{if } \max H_0 < 1\ , \\ 0 & \text{if } \min H_0 < 1, \max H_0 \geq 1\ , \end{cases} \tag{4.17}$$

$$\text{Final } (H_S + H_R) = \begin{cases} H_S + H_R & \text{if } \min\left(H_S + H_R\right) \geq 1\ , \\ 0 & \text{if } \max\left(H_S + H_R\right) < 1\ , \\ 0 & \text{if } \min\left(H_S + H_R\right) < 1, \max\left(H_S + H_R\right) \geq 1\ . \end{cases} \tag{4.18}$$

When $\max\ H_0 < 1$ and/or $\max\ (H_S{+}H_R) < 1$, then the host populations are less than 1 individual, so it is biologically reasonable to consider them as extinct,

which leads to extinction of the parasitoid population. If $\min\ H_0 < 1$ but $\max\ H_0 \geq 1$ and/or $\min\ (H_S{+}H_R) < 1$ but $\max\ (H_S{+}H_R) \geq 1$ then the population oscillates below 1 individual and it is biologically reasonable to consider them as extinct.

In cases where the final infected and uninfected host populations are both not extinct, Kwiatkowski and Vorburger (2012) defined the average fraction of infected hosts in the total host population, μ, as

$$\mu = \frac{H_S + H_R + V_S}{H_0 + H_S + H_R + V_0 + V_S} \ . \tag{4.19}$$

This quantity was modified to consider the average fraction of available infected hosts in the total available host population:

$$\alpha = \frac{H_S + H_R}{H_0 + H_S + H_R} \ . \tag{4.20}$$

α can take the following values:

$$\alpha = \begin{cases} \text{Undefined} & \text{if Final} H_0 + \text{Final}\,(H_S + H_R) = 0 \ , \\ 0 & \text{if Final} H_0 \neq 0, \text{Final}\,(H_S + H_R) = 0 \ , \\ 1 & \text{if Final} H_0 = 0, \text{Final}\,(H_S + H_R) \neq 0 \ , \\ \frac{H_S + H_R}{H_0 + H_S + H_R} & \text{if Final} H_0 \neq 0, \text{and Final}\,(H_S + H_R) \neq 0 \ . \end{cases} \tag{4.21}$$

Table 4.3: Using final population values to determine biologically reasonable outcomes for population dynamics

Host population outcome:	**α value:**
Uninfected and infected hosts extinct	Undefined
Infected hosts extinct	0
Uninfected hosts extinct	1
Uninfected and infected hosts coexist	$\frac{H_S + H_R}{H_0 + H_S + H_R}$

4.4.8 Costs versus benefits parameter sweeps

Kwiatkowski and Vorburger (2012) carry out a number of sweeps across two key parameters: the constitutive cost of harbouring an endosymbiont infection, c_c, and the strength of protection provided by the endosymbiont infection, p_s. For a number of possible pairs of parameter values, they calculate the average fraction of infected hosts in the host population and use this to generate plots using the value of the average fraction to determine the colour of the area in the plot adjacent to the point. As will become apparent in the results section, red is used to show an outcome where infected hosts have prevailed over uninfected hosts; blue is used to show an outcome where uninfected hosts have prevailed over infected hosts. Uninfected and infected hosts coexisting (0 $<$ average fraction of infected hosts $<$ 1) take intermediate colours as dictated by the MATLAB colormap in use. Code was written in MATLAB to carry out similar sweeps across parameter space. The range of possible values for each parameter ($0 \leq c_c \leq 1$ and $0 \leq p_s \leq 1$) was divided by the resolution of the sweep to create a grid with (x,y) coordinates (c_c', p_s') as shown in figure 4.7. The average fraction of *available* hosts, α, was calculated for each grid point and the corresponding adjacent grid square coloured according to numerical value as described above. To allow for comparison with the results of Kwiatkowski and Vorburger (2012), the same colormap was used. For each grid point, the numerical values of the quantities listed in figure 4.7 were calculated and recorded for subsequent analysis.

4.4.9 Stability and coexistence boundaries

The boundary between stable and oscillating populations was found computationally using equation 4.16. In the revised model, the cut-off value for stability was determined to be ≤ 0.0001. The numerical quantity std $\left[\frac{P}{\text{mean}(P)}\right]$ was calculated for each grid point in a $c_c \times p_s$ parameter space as indicated in figure 4.7 and the "contour" command used in MATLAB to plot the boundary.

The boundary between coexistent and extinct populations was also found computationally using the values for Final H_0 and Final $(H_S + H_R)$ (equations 4.17 and 4.18) assigned at each grid point in a $c_c \times p_s$ parameter space as indicated in figure 4.7, and the "contour" command again used in MATLAB to plot boundaries.

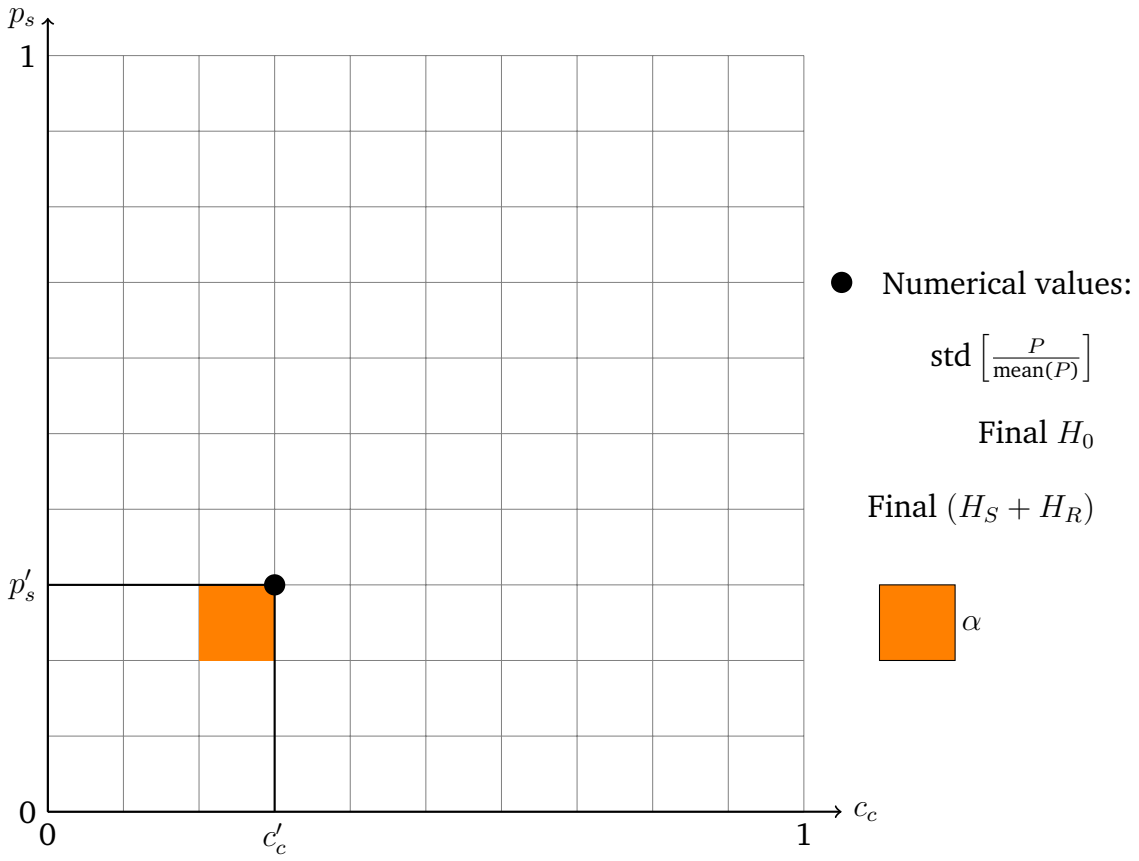


Figure 4.7: Parameter sweep - costs and benefits of endosymbiont infection.

4.4.10 Depression of host populations by parasitoids

It is possible to calculate, as a model output at each grid point, the extent to which the total host equilibrium population (infected and uninfected) is held below carrying capacity, q, (equation 4.22, (Hassell, 1978)) where

$$q = \frac{\text{mean } H_0 + \text{mean } H_S + \text{mean } H_R}{k} \,. \tag{4.22}$$

This measure relates to the effectiveness of the parasitoid wasps as natural enemies. In turn, this links the costs and benefits of infection and the fractions of uninfected and infected hosts in the host population to the overall host-parasitoid dynamics. By comparing q against the stability of and either coexistence or extinction of host populations for the costs and benefits of endosymbiont infection, it is possible to identify the parameter range corresponding to stable coexistence of uninfected and infected host populations.

4.4.11 Horizontal and vertical transmission parameter sweeps

Kwiatkowski and Vorburger (2012) carry out a number of simulations to determine the size of the coexistence region of $c_c \times p_s$ parameter space at different values of c_i across $t_h \times t_v$ parameter space. The parameters t_h and t_v were varied in turn, i.e. one parameter fixed whilst the other parameter varied for each different c_i, and the proportion of parameter space corresponding to coexistence computed relative to the whole parameter space. For this study, using similar methodology to that described in section 4.4.8, the range of possible values for each parameter ($0.0 \leq t_h \leq 0.001$ and $0.8 \leq t_v \leq 1.0$) was divided to create a grid with (x,y) coordinates (t'_h, t'_v) as shown in figure 4.8. The average fraction of the coexistent $c_c \times p_s$ parameter grid-spaces relative to the total grid spaces was calculated and recorded for each grid point by performing a sweep through $c_c \times p_s$ parameter space for the t'_h, t'_v and c_i input values. This fraction was plotted on the z-axis to create a 3-dimensional plot of the variation in coexistence for $t_h \times t_v$ parameter space at different c_i.

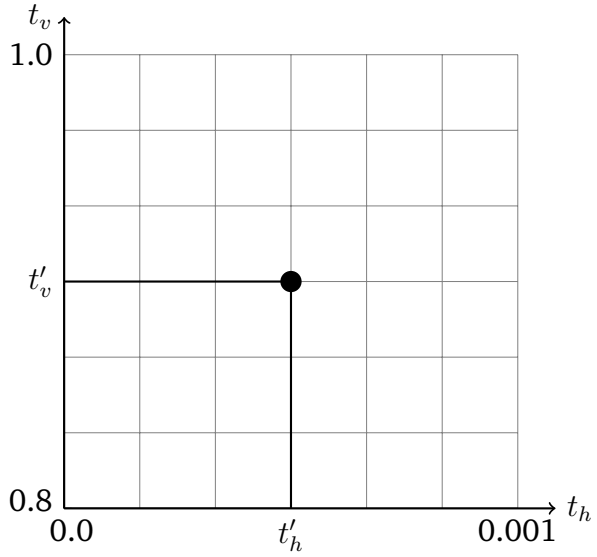


Figure 4.8: Parameter sweep - horizontal and vertical endosymbiont transmission

4.5 Modelling "all-or-none" encapsulation

Code was written in MATLAB to simulate the population dynamics described by Godfray and Hassell (1991) (equation 1.8). The computational method developed

to find the stability boundaries described in section 4.4.9 was used to investigate further the stability properties of this model.

The model equations with a type I functional response are:

$$\left.\begin{array}{rcl} H(t+1) & = & H(t)\lambda\left[\exp\left(-aP(t)\right)+\eta\left(1-\exp\left(-aP(t)\right)\right)\right] \ , \\ P(t+1) & = & H(t)\left(1-\eta\right)\left(1-\exp\left(-aP(t)\right)\right) \ . \end{array}\right\} \tag{4.23}$$

Incorporating a type II functional response into the model:

$$\left.\begin{array}{rcl} H(t+1) & = & H(t)\lambda\left[\exp\left(\frac{-aP(t)}{1+abH(t)}\right)+\eta\left(1-\exp\left(\frac{-aP(t)}{1+abH(t)}\right)\right)\right] \ , \\ P(t+1) & = & H(t)\left(1-\eta\right)\left(1-\exp\left(\frac{-aP(t)}{1+abH(t)}\right)\right) \ . \end{array}\right\} \tag{4.24}$$

Code was then written in MATLAB to investigate the population outcomes for a mutant clone invading a resident clone when host population density dependence was present as postulated by Godfray and Hassell (1991). The encapsulation ability and fecundity of the resident clone, η_1 and λ_1 respectively, are fixed; the encapsulation ability and fecundity of the mutant clone, η_2 and λ_2 respectively, are varied. The population outcome is assessed either as clonal line "winning" with the other clonal line going extinct or as both clonal populations coexisting. Coexisting population dynamics may be stable or oscillatory.

The model equations for the evolution of encapsulation (with resource limitation) are:

$$\left.\begin{array}{rcl} N(t+1) & = & N(t)\lambda_1\exp\left(-\frac{(N(t)+S(t))}{k}\right)\times \\ & & \left[\exp\left(-aP(t)\right)+\eta_1\left(1-\exp\left(-aP(t)\right)\right)\right] \ , \\ S(t+1) & = & S(t)\lambda_2\exp\left(-\frac{(N(t)+S(t))}{k}\right)\times \\ & & \left[\exp\left(-aP(t)\right)+\eta_2\left(1-\exp\left(-aP(t)\right)\right)\right] \ , \\ P(t+1) & = & N(t)\left(1-\eta_1\right)\left[1-\exp\left(-aP(t)\right)\right]+ \\ & & S(t)\left(1-\eta_2\right)\left[1-\exp\left(-aP(t)\right)\right] \ . \end{array}\right\} \tag{4.25}$$

As previously, population oscillations were determined by looking for oscillations in the wasp population. Coexistence was determined by comparison of final population sizes whereby biologically unrealistic final population sizes of less than 1 were considered extinct. MATLAB pseudocolor plots of "resident' or "mutant" host population size against encapsulation ability and fecundity to identify host popu-

lation coexistence and extinction and a binary response determined from the ratio std $\left[\frac{P}{\text{mean}(P)}\right]$ against encapsulation ability and fecundity to determine final population dynamics were generated using results from parameter sweeps based on the code written to generate parameter sweeps in the Kwiatkowski and Vorburger (2012) model. The $c_c \times p_s$ parameter space in the Kwiatkowski and Vorburger (2012) model can be compared to the $\lambda \times \eta$ parameter space in the Godfray and Hassell (1991) model.

5 Modelling Results

5.1 Introduction

In chapter 4, a discrete-time model of the host-parasitoid dynamic of a *A. pisum-A. ervi* system was presented. The model arose from development and refinement of the modelling approach taken by Kwiatkowski and Vorburger (2012). The criteria for interpreting the resulting host–parasitoid dynamics were explained and the general form of the graphical output plots of sweeps through parameter space explained. Godfray and Hassell (1991) developed models to explore the consequences of encapsulation on host-parasite dynamics. This chapter brings together the work of Kwiatkowski and Vorburger (2012) and Godfray and Hassell (1991) using the model presented in section 4.4.5.

5.2 Results of numerical simulations

5.2.1 Comparing the new model with the Kwiatkowski and Vorburger (2012) model

Section 4.3.3 highlighted an anomaly with the Kwiatkowski and Vorburger (2012) model coding whereby changing the initial population sizes affected the resulting population dynamics. Unlike the Kwiatkowski and Vorburger (2012) code, the dynamics of the model refined and coded in this study do not change when initial population sizes are varied. The results of two sets of simulations are shown in figures 5.1 and 5.2. In these simulations, the sizes of the initial uninfected and infected populations were set at $H_0 = 8000$, $H_S = 2000$ and $P = 200$ for three different values for the strength of protection conferred by the endosymbiont infection $p_s = 0.90$, $p_s = 0.75$ and $p_s = 0.50$, and then reversed to $H_0 = 2000$, $H_S = 8000$ and $P = 200$ for the same three values of p_s. Within each set of simula-

tions, the host population outcomes remained stable coexistence when $p_s = 0.90$, oscillatory coexistence when $p_s = 0.75$ and unprotected (uninfected) hosts only existing when $p_s = 0.50$. In the case of stable coexistence, the size of the resulting uninfected host population was $H_0 = 26.47$, as indicated by the coordinates of the highlighted point. In the case where uninfected hosts only persist long term, the population size of the infected host population was $H_0 = 0.07$, as indicated by the coordinates of the highlighted point; as this population size is less than one individual, it is biologically realistic to regard the infected host population as going extinct.

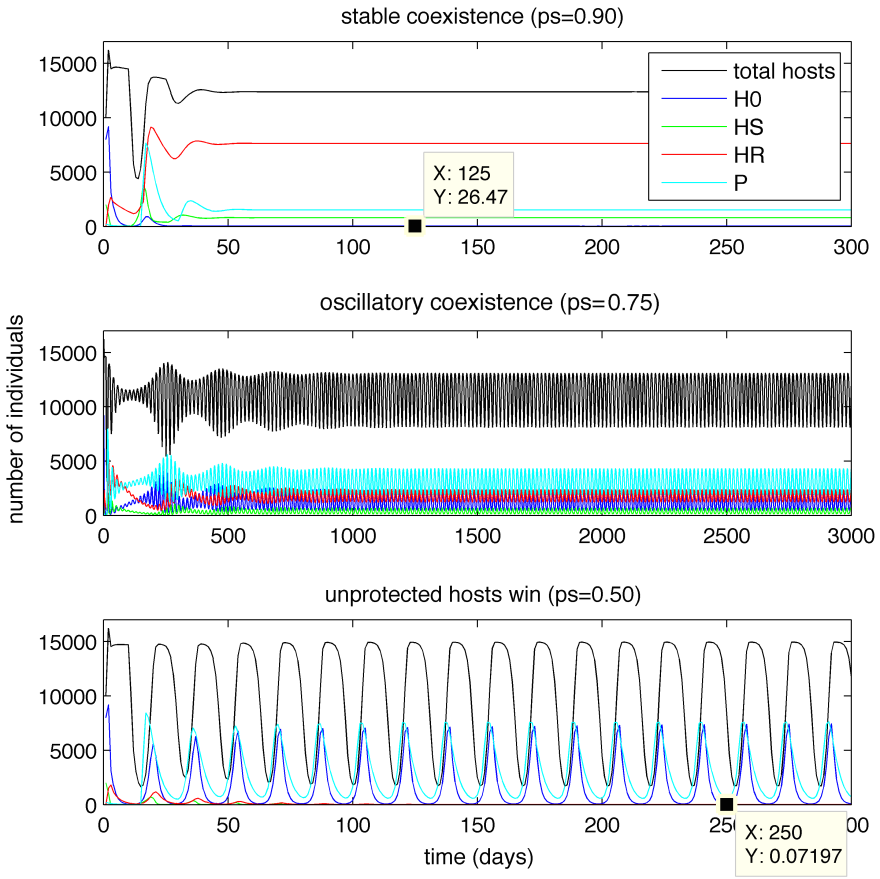


Figure 5.1: Results of simulation to look at effect on population outcomes using new model code with initial population sizes $H_0 = 8000$, $H_S = 2000$, $P = 200$ with varying strengths of protection conferred by the endosymbiont.

A further anomaly revealed in section 4.3.3 concerned abrupt changes in long

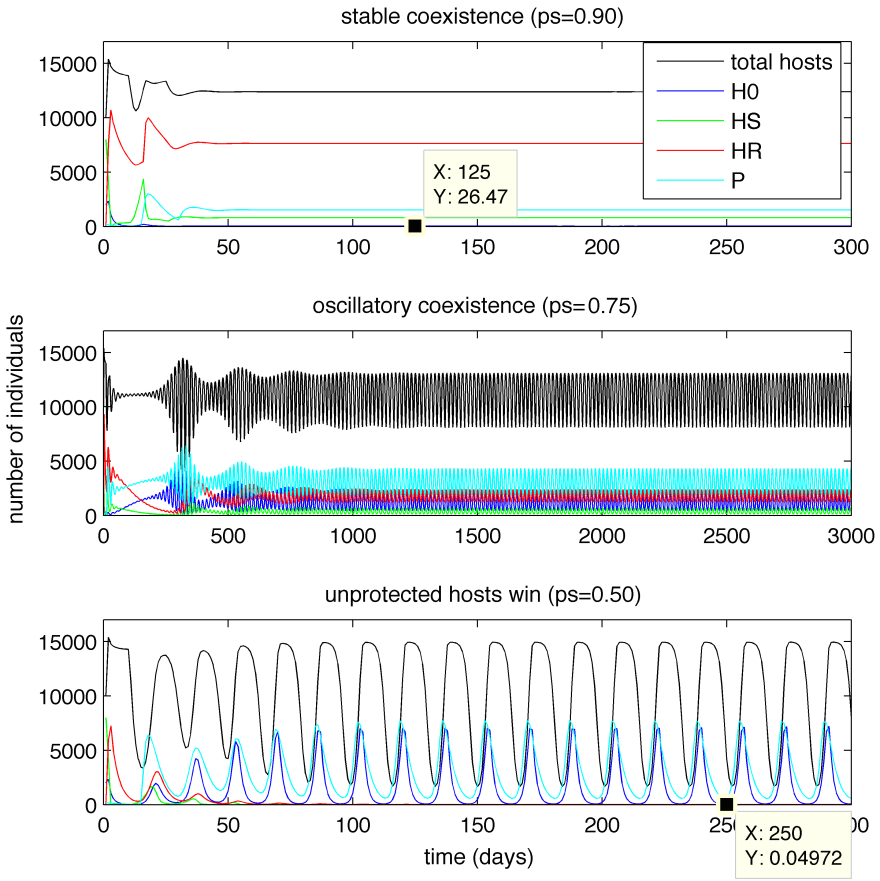


Figure 5.2: Results of simulation to look at effects on population outcomes using new model code with initial host populations sizes reversed to $H_0 = 2000$, $H_S = 8000$, $P = 200$ with varying strengths of protection conferred by the endosymbiont.

term population dynamics with only small changes in the sizes of the time delays in the model. The time delays are determined by the parasitoid development parameters l_k and l_e (time taken to kill and to emerge from a parasitised aphid host respectively). This was illustrated by the results of a set of simulations shown in figure 4.4. The simulations were repeated using the new model code. Figure 5.3 shows that an increase in the time delays by 1 day ($l_k = 6$ and $l_e = 10$) does not sharply force the population dynamics into stable coexistence when $p_s = 0.75$ but that oscillatory coexistence remains.

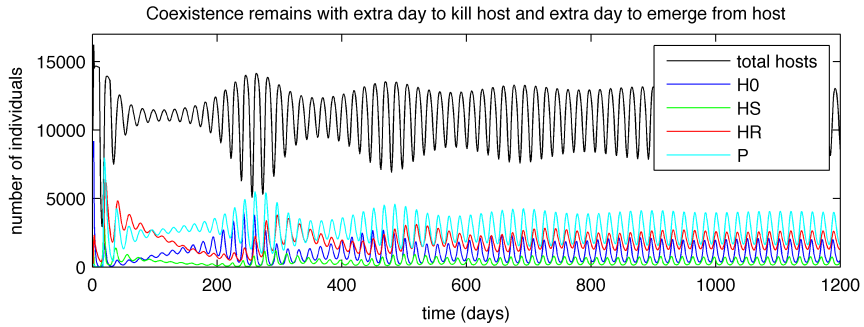


Figure 5.3: Results of simulation using the new model code to explore the effects on the final population outcome by increasing the time that a wasp takes to kill and to emerge from an aphid host by one day to $l_k = 6$ and $l_e = 10$.

5.2.1.1 Induced costs to pea aphids of harbouring an endosymbiont infection

Figure 5.4 reviews, from section 4.4.8, the colour of the pseudocolor plot in relation to the host population outcomes for sweeps through $c_c \times p_s$ parameter space using both the Kwiatkowski and Vorburger (2012) and new model code. Recall that the benefits of harbouring a secondary endosymbiont infection are expressed through the strength of protection parameter, p_s, and the costs of infection expressed through the constitutive cost and induced cost parameters, c_c and c_i respectively. Each square in the Kwiatkowski and Vorburger (2012) plot depicts the long-term fraction of infected (protected) hosts in the total host population, μ. Each square in this pseudocolor plot shows the results of simulations using the new model depicting the long-term fraction of *available* infected (protected) hosts in the total host population, α. At the extremes of the colormap used in the plots, dark red and dark blue denote uniform protected and unprotected host populations respectively; intermediate rainbow colours result from parameter values where coexistence is possible as shown in figure 5.4. Note that very small populations sizes may not be distinguishable by eye from extinct populations by simply looking at these coloured sweeps, but that these plots should be used in conjunction with the stability and coexistence boundary plots described in section 4.4.9.

Figure 5.5 shows the results of a simulation carried out using the code provided by Kwiatkowski and Vorburger (2012) using the parameters from their paper (table 4.2). It is included here to allow comparison between results obtained by Kwiatkowski and Vorburger (2012) and results from a simulation carried out us-

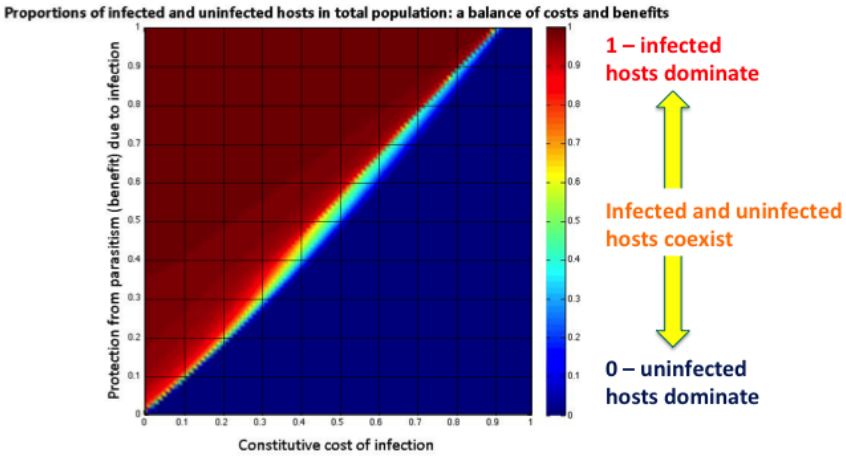


Figure 5.4: Illustration of how the colours in a typical pseudocolor plot of the results from a sweep through $c_c \times p_s$ parameter space relate to host population outcomes.

ing the MATLAB code for the refined model developed in this study. The results of this latter simulation are shown in figure 5.6.

The method described in section 4.4.9 was used to determine the boundaries between stable and oscillatory final population outcomes, and also between persistence of the uninfected host population, H_0, only and also the coexistence of the uninfected and infected host populations, $H_S + H_R$.

Simulations (figure 5.8) were carried out using the set of default parameters in the Kwiatkowski and Vorburger (2012) study with the exception of the constitutive cost that was kept constant at $c_c = 0.5$ and the strength of protection provided by the endosymbiont varied from low ($p_s = 0.25$) to medium ($p_s = 0.5$) to high ($p_s = 0.75$). With this midrange constitutive cost, a low strength of protection caused the infected hosts to go extinct whilst uninfected hosts and parasitoid wasp populations persisted in an oscillatory manner. With both the constitutive cost and strength of protection midrange, populations coexist in an oscillatory manner with the uninfected host population dominating the infected host population. With a high strength of protection and a midrange constitutive cost, all populations coexist in a stable manner. However, the size of the uninfected host population in this region of stable coexistence is small. These results confirm the boundaries between stable and oscillatory final population outcomes, and also between persistence of the uninfected host population only and coexistence of both uninfected and infected host populations shown in figure 5.7.

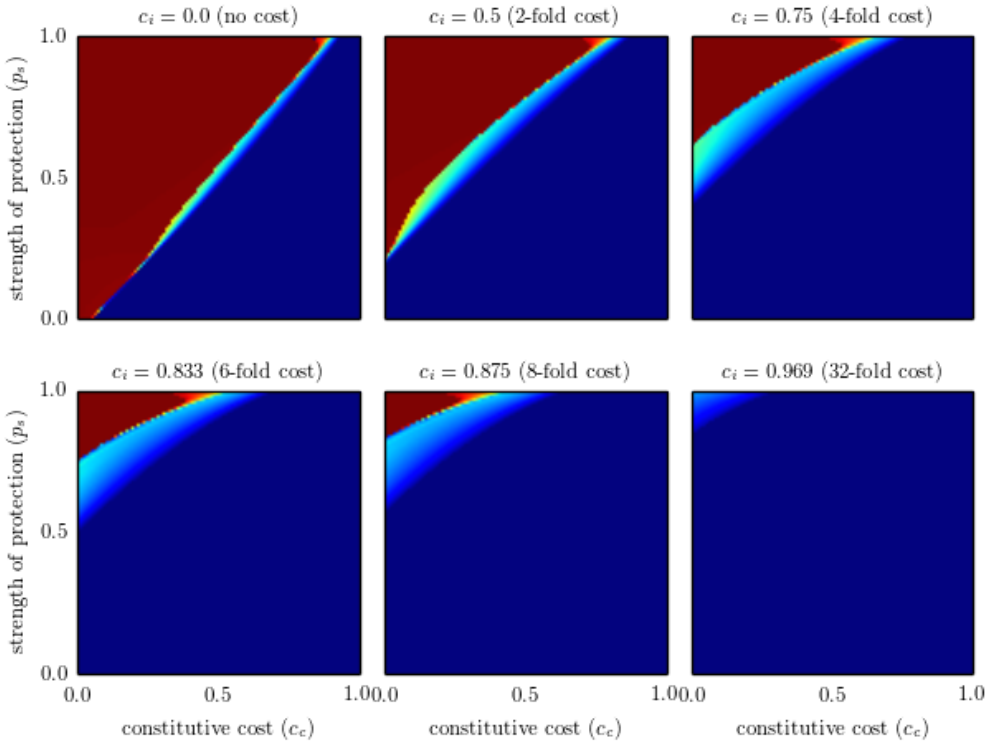


Figure 5.5: Using Kwiatkowski and Vorburger (2012) model code to replicate their sweep across $c_c \times p_s$ parameter space for differing induced costs of harbouring secondary endosymbiont infection.

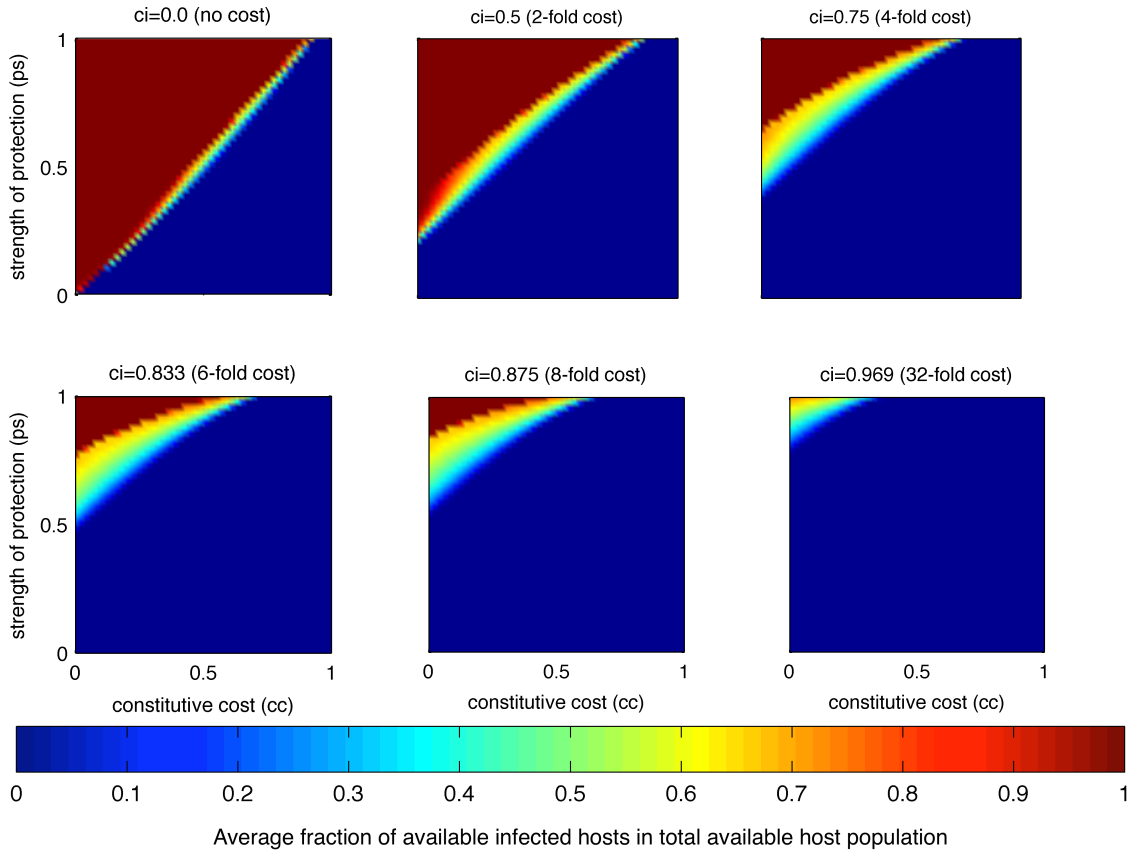


Figure 5.6: Result of simulations using new model code to replicate Kwiatkowski and Vorburger (2012) sweep across $c_c \times p_s$ parameter space (figure 5.5) for differing induced costs of harbouring secondary endosymbiont infection.

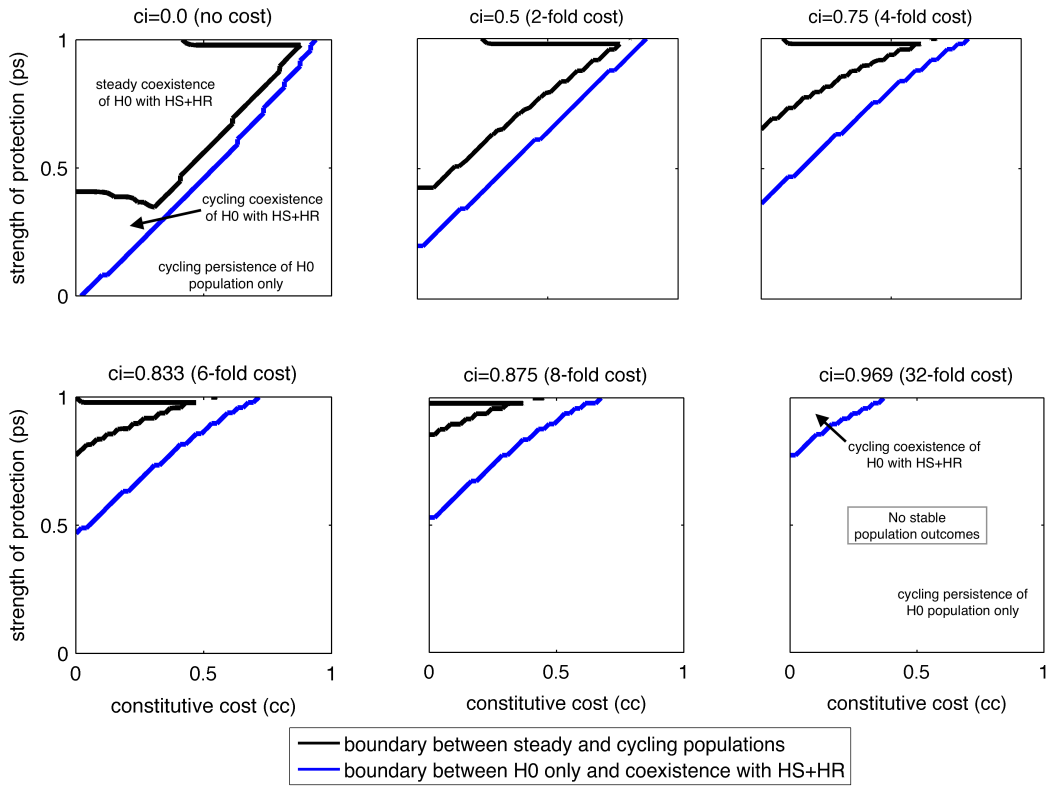


Figure 5.7: Result of simulations to determine the boundaries between stable and oscillatory final population outcomes and between persistence of the uninfected host population, H_0, only and coexistence of the uninfected and infected host populations, $H_S + H_R$, in the $c_c \times p_s$ parameter space with the new model as shown in figure 5.6.

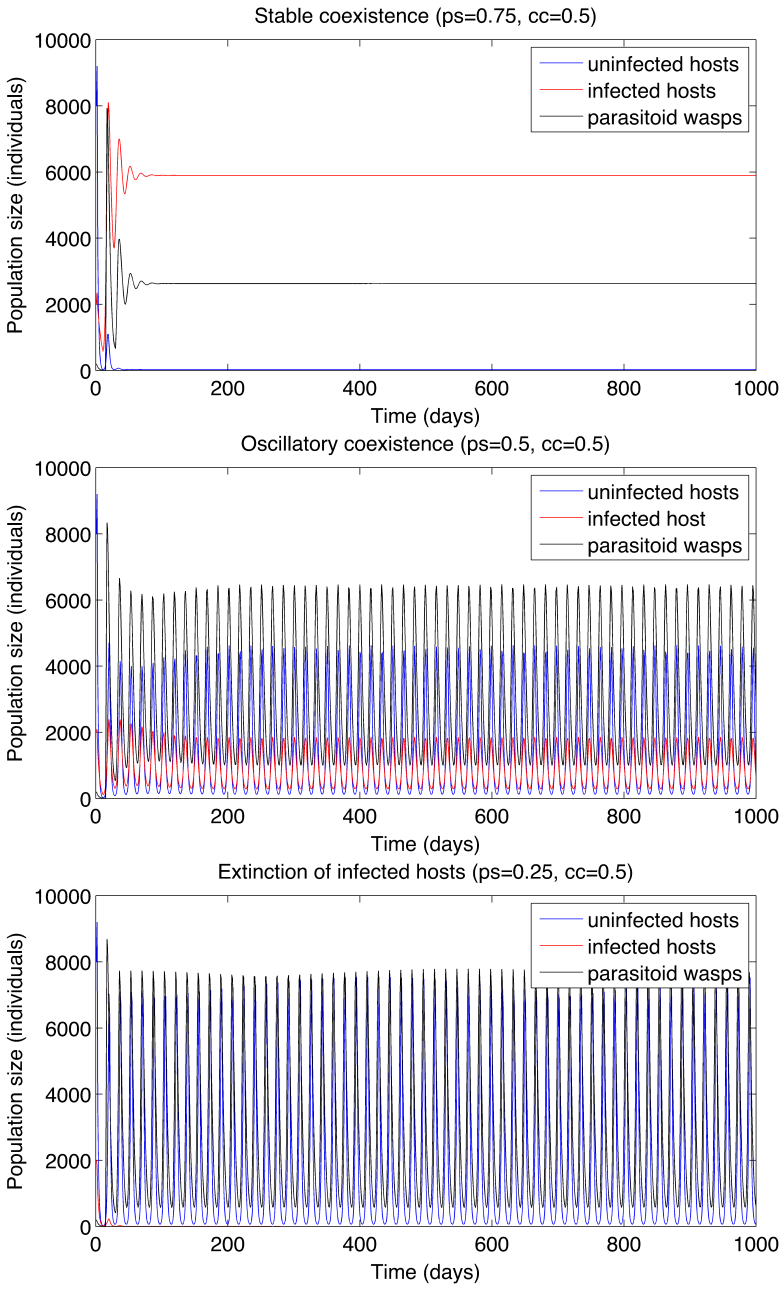


Figure 5.8: Results of simulations showing the different population dynamics obtained for low, medium and high strength of protection provided by the endosymbiont.

Comparing the two sets of sweeps (figures 5.5 and 5.6) through $c_c \times p_s$ parameter space for increasing induced cost, c_i, of harbouring a secondary endosymbiont, several differences can be seen. Firstly, the region $c_c \times p_s$ parameter space in which coexistence of both host populations takes place is much larger for the new

model developed in this study. Secondly, for induced costs of zero, there is a difference in the lower left area on the plots. Using the new model, the boundary between existence of uninfected hosts only and coexistence of host populations passes approximately through the origin ($p_s = 0$, $c_c = 0$), whereas, in the study by Kwiatkowski and Vorburger (2012), a boundary between persistence of infected hosts only and persistence of uninfected hosts only passes through approximately $p_s = 0$ and $c_c = 0.1$. The output dynamics from both models illustrating this difference at very low constitutive costs and strength of protection are shown in figure 5.9. Thirdly, whereas the initially very narrow range of cost-benefit values that result in coexistence in the Kwiatkowski and Vorburger (2012) model (shown by the narrow bands of intermediate colours in figure 5.5) increases with increasing induced cost of harbouring a secondary endosymbiont infection, c_i, results from the simulations carried out with the new model (figure 5.7) show a decrease in the initially much larger range of cost-benefits values promoting coexistence. However, the broad trend of uninfected host dominance spreading to lower cost, higher benefit parameter values as the induced cost increases holds true for both models. This region of uninfected host dominance is shown by the dark blue areas in figures 5.5 and 5.6.

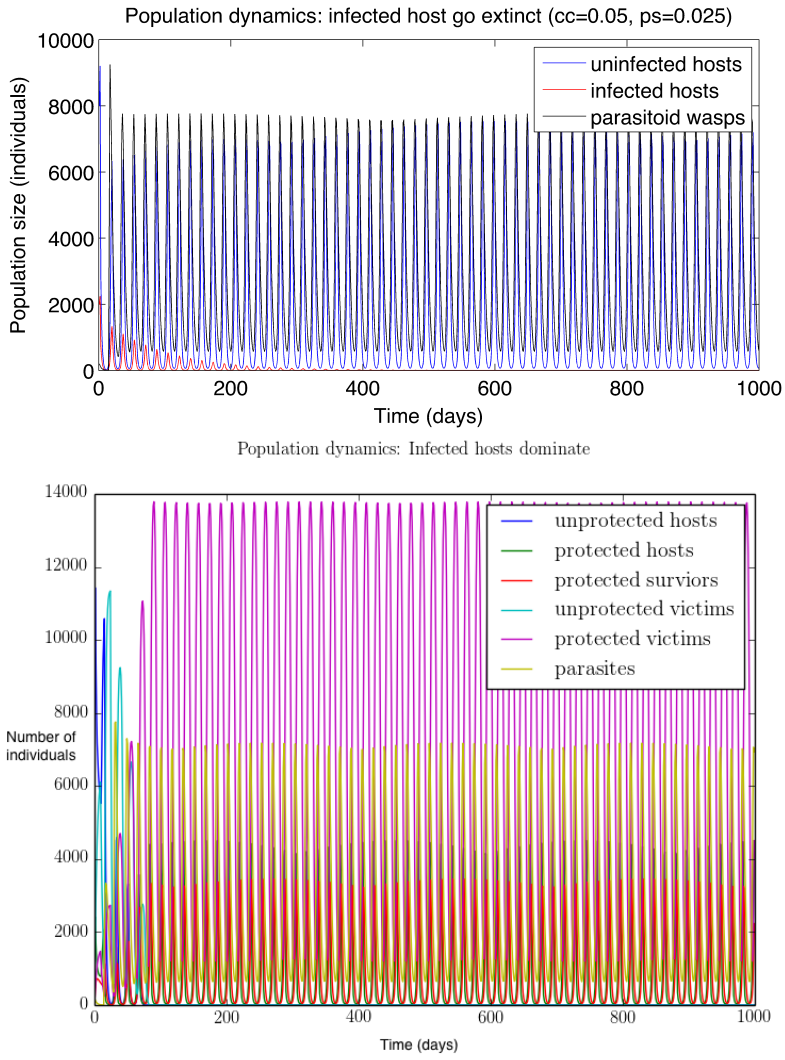


Figure 5.9: Results of simulations comparing the Kwiatkowski and Vorburger (2012) model output (below) and new model output (above) for very low constitutive costs and strength of protection provided by the endosymbiont.

5.2.2 Time delays due to parasitoid wasp life history traits

With parameters set to default values from Kwiatkowski and Vorburger (2012) (table 4.2) with the exception of $p_s = 0.75$, $c_c = 0.2$ and $c_i = 0.75$, simulations were carried out with varying l_k and l_e to show the effects of increasing the time taken for a parasitised host to be killed by parasitism and the time taken for wasp offspring to emerge from a parasitised host. The total time delays used in the simulations were $l_k + l_e = 0 + 0 = 0$ d, $l_k + l_e = 2 + 2 = 4$ d, $l_k + l_e = 4 + 2 = 6$ d, $l_k + l_e = 6 + 3 = 9$ d, $l_k + l_e = 9 + 5 = 14$ d and $l_k + l_e = 15 + 10 = 25$ d. The results from these simulations (figures 5.10, 5.11 and 5.12) show that increasing these time delays leads to oscillations in the system. The increasing time delays also decrease the total host population size and change the dominant host population from uninfected to infected. As previously stated in section 4.4.4, development time from egg to emergent adult wasp typically takes two weeks at $21\,^{\circ}$C (Bai, 1991) so the case where $l_k + l_e = 9 + 5 = 14$ d is the most biologically realistic of these simulations for this study system.

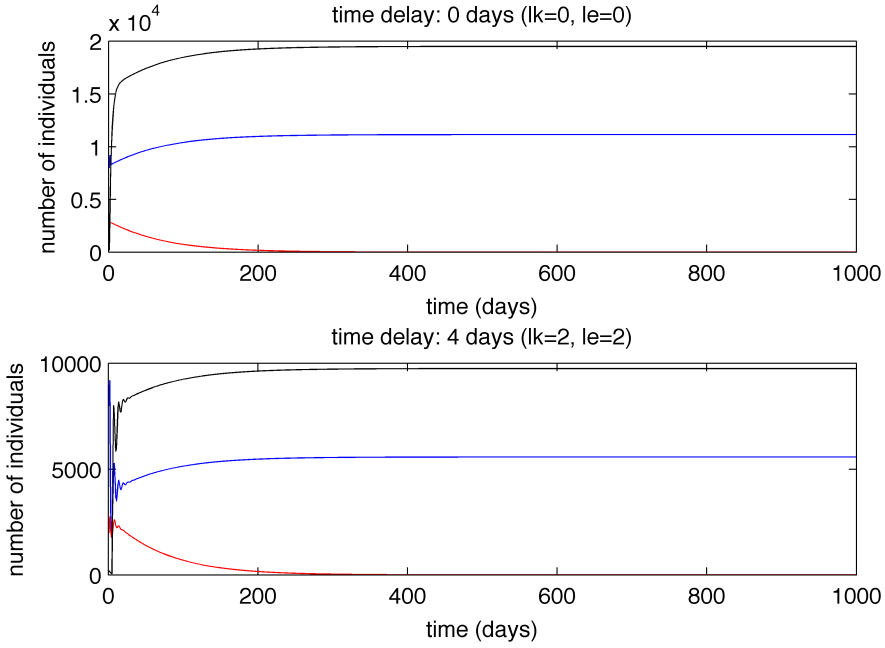


Figure 5.10: Results from simulations exploring the effect on population sizes and stability of increasing time delays, l_k and l_e, from zero to 4 d with new model code.

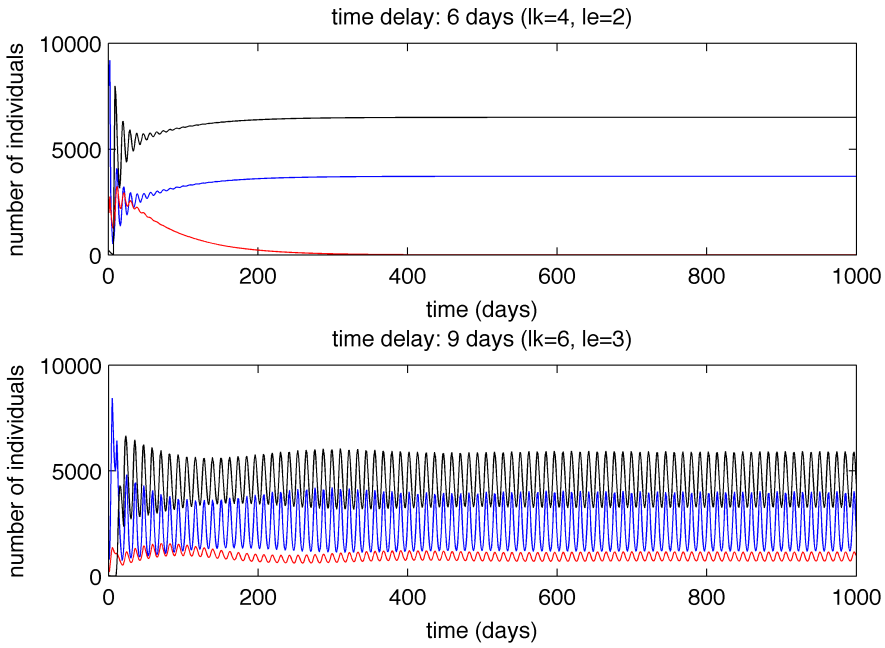


Figure 5.11: Results from simulations exploring the effect on population sizes and stability of increasing time delays, l_k and l_e, from $6\,\mathrm{d}$ to $9\,\mathrm{d}$ with new model code.

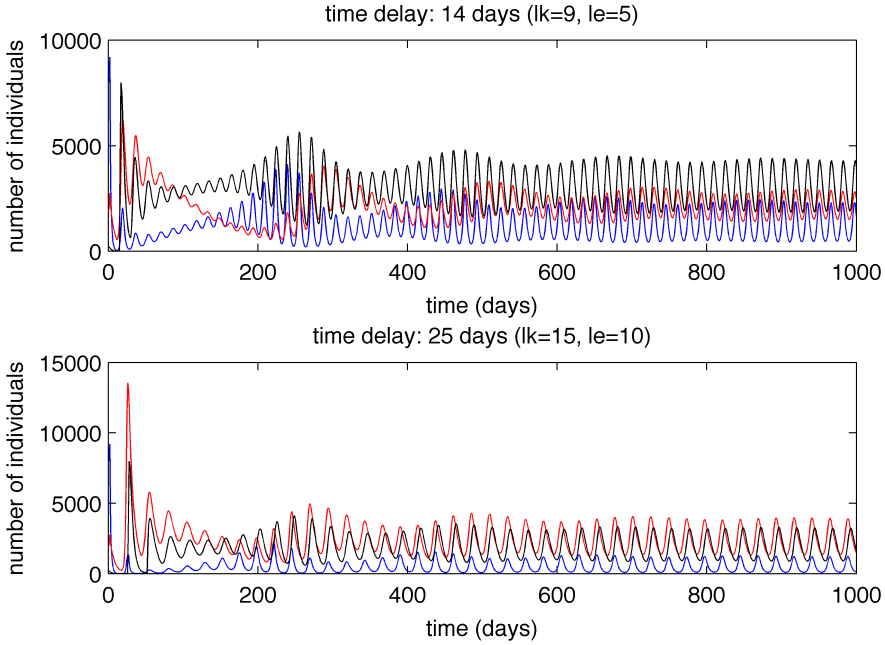


Figure 5.12: Results from simulations exploring the effect on population sizes and stability of increasing time delays, l_k and l_e, from $14\,\mathrm{d}$ to $25\,\mathrm{d}$ with new model code.

5.2.3 Stability boundaries and transmission rates

A number of simulations were carried out using the new model to identify the boundary between stable and oscillating final population outcomes for a range of vertical (maternal) transmission rates and horizontal (conspecific) transmission rates. The results of these simulations are shown in figures 5.13 and 5.14 respectively and show the general trends resulting from a change in these parameter values.

When the rate of vertical transmission is increased from $t_v = 0.8$ to $t_v = 1.0$, the stability boundary shifts to lower values of the strength of protection, p_s for the same values of constitutive cost, c_c (figure 5.13). This shift is not uniform but is least for higher values of c_c and greatest for values of $c_c \approx 0.3$. In these simulations, the rate of horizontal transmission, t_h, was held at a value of $t_v = 0.001$ and the induced cost, c_i, was zero.

When the rate of horizontal transmission is increased from $t_h = 0.0$ to $t_h = 0.001$, the stability boundaries do not noticeably change position within the computational limits of the model and, unlike figure 5.13, the lines on the graph corresponding to each value of t_h overlap. In order to assess if any change in position of the stability boundaries due to a change in t_h occurred for any biologically reasonable value of t_v, the simulations were repeated across the range $0.8 \leq t_v \leq 1.0$ and the results are shown in figure 5.14. As expected, the changes in vertical transmission rate shifts the stability boundaries showing the same trend as in figure 5.13 but, within each simulation, the change in horizontal transmission rate does not affect the position of the stability boundary.

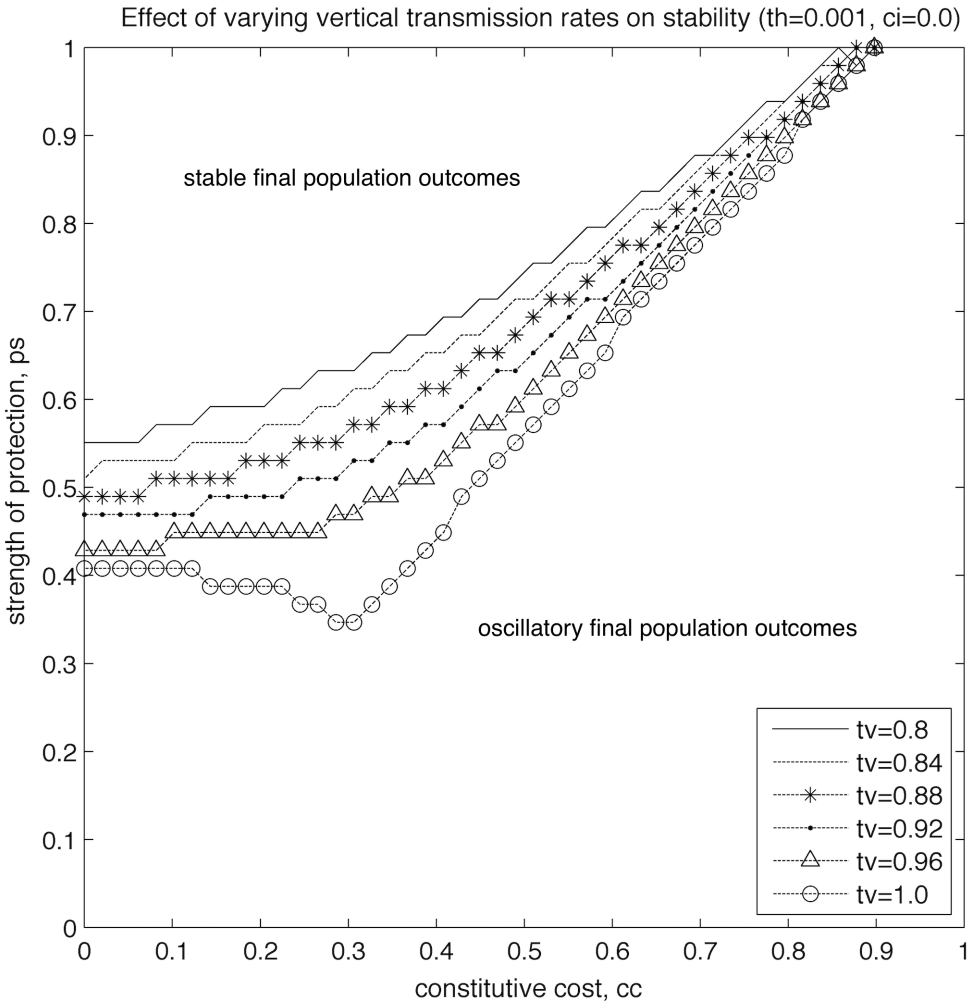


Figure 5.13: Results of simulations exploring the effect of changing vertical transmission rate, t_v, on the boundary between stable and oscillatory final population outcomes.

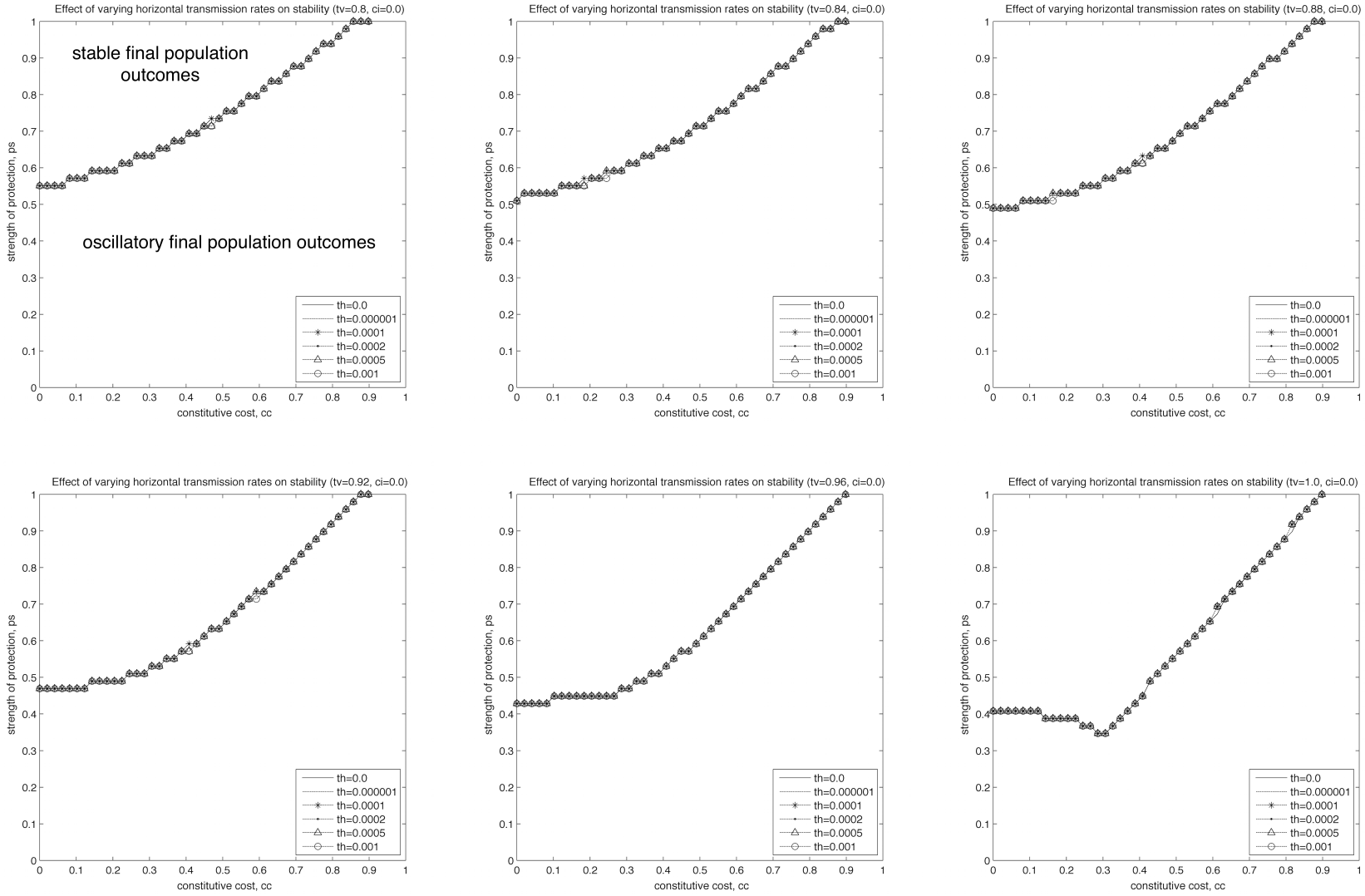


Figure 5.14: Results of simulations exploring the effect of changing horizontal transmission rate, t_h, on the boundary between stable and oscillatory final population outcomes.

Section 1.5.5 relates encapsulation to the safety of a partial refuge from predation. In the model, hosts gaining a protective secondary endosymbiont infection are analogous to hosts becoming safe from predation; hosts losing the protective infection become liable to predation again. The terms in the model equations presented in section 4.4.3 describing this are:

Horizontal transmission **in** to partial refuge

$$= \left[1 - \exp\left\{-\frac{t_h(H_S(t) + H_R(t) + V_S(t))}{H_0(t) + H_S(t) + H_R(t) + V_0(t) + V_S(t)}\right\}\right] H_0(t).$$

Vertical transmission **out** from partial refuge

$$= (1 - t_v)(1 - c_c)\left[b_h - \frac{(b_h - d_h)(H_0(t) + H_S(t) + H_R(t) + V_0(t) + V_S(t))}{k}\right] H_S(t) +$$

$$(1 - t_v)(1 - c_c)(1 - c_i)\left[b_h - \frac{(b_h - d_h)(H_0(t) + H_S(t) + H_R(t) + V_0(t) + V_S(t))}{k}\right] H_R(t).$$

In general terms:

$$\text{Horizontal transmission } \textbf{in} \text{ to partial refuge} \quad \propto \quad -\exp(-t_h), \tag{5.1}$$

$$\text{Vertical transmission } \textbf{out} \text{ from partial refuge} \quad \propto \quad -t_v. \tag{5.2}$$

A simulation was carried out using the method described in section 4.4.11 to look at the effect on the size of the region of the coexistence in $c_c \times p_s$ parameter space across $t_h \times t_v$ parameter space for the new code. This simulation consisted of a number of $c_c \times p_s$ sweeps with all parameters held constant at the Kwiatkowski and Vorburger (2012) default values to allow for comparison with their results, except for c_c and p_s (varied within each sweep) and t_h and t_v (varied between sweeps). Note the use of a log scale on the horizontal transmission axis. The size of the induced cost, c_i, in this simulation was zero and the strength of endosymbiont protection provided by the secondary endosymbiont was the same for second and subsequent parasitoid attacks. The results show that looking across the grid holding the vertical transmission rate, t_v, constant but varying the horizontal transmission rate, t_h, shows no discernible change in the amount of coexistence. However, varying the vertical transmission rate, t_v, whilst keeping the horizontal transmission rate, t_h, constant shows that the fractional amount of coexistence increases slightly up to ≈ 0.3 when $t_v \approx 0.95$ before decreasing sharply as t_v ap-

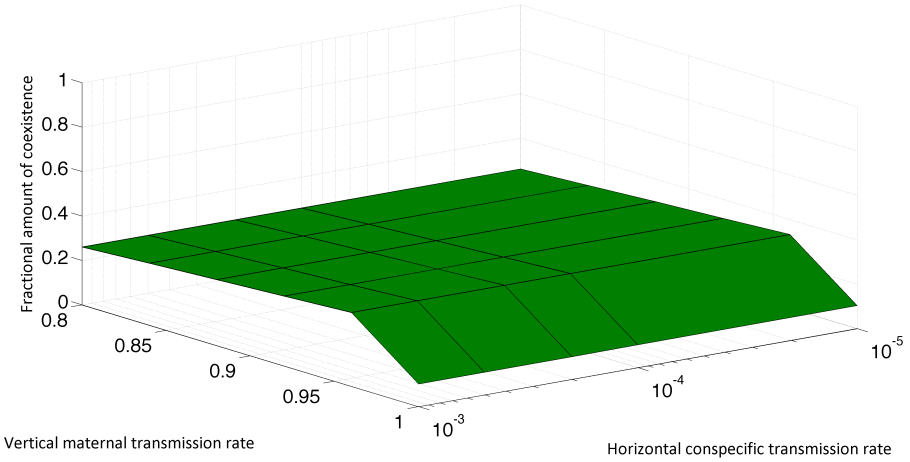


Figure 5.15: Results of simulation exploring the effect of changing horizontal transmission rate, t_h, and vertical transmission rate, t_v, on the size of the coexistence region of $c_c \times p_s$ parameter space.

proaches 1. These trends are in broad agreement with the general results of the simulations shown in figures 5.13 and 5.14, whereby changing the vertical transmission rate, t_v, affects the position of the stability boundary, but changing the horizontal transmission rate, t_h, does not.

The method described in section 4.4.9 was again used to determine the boundaries between persistence of the infected host population, $H_S + H_R$, only or uninfected host population, H_0, only and coexistence of the uninfected and infected host populations for changing horizontal, t_h, and vertical, t_v, transmission rates. Simulations were carried out for 6 incrementally increasing pairs of values of t_h and t_v, equivalent to a diagonal cross-section across the 3D plot of fractional coexistence against t_h and t_v shown in figure 5.15. The values of t_h and t_v used were $t_h = 0$, $t_v = 0.8$; $t_h = 0.00001$, $t_v = 0.84$; $t_h = 0.0001$, $t_v = 0.88$; $t_h = 0.0002$, $t_v = 0.92$; $t_h = 0.0005$, $t_v = 0.96$ and $t_h = 0.001$, $t_v = 1$. Results are shown in figure 5.16. The boundary between existence of uninfected hosts and coexistence of uninfected with infected host populations is shown in blue; the boundary between coexistent uninfected with infected host populations and infected populations only is shown in red. The size of the coexistence region to the upper left of the blue coexistence boundary and beneath any red boundary divided by the total plot area gives the amount of fractional coexistence. From comparison with figure 5.14, it can be inferred from the plots that the possible final outcomes for

the host population dynamics are stable coexistence of uninfected and infected populations, oscillatory coexistence of uninfected and infected populations, stable and oscillatory persistence of the infected population only and, lastly, oscillatory persistence of the uninfected population only. Further simulations (not shown) show that persistence of the uninfected population only occurs, for the same set of parameter values, when $0.9996 \leq t_h \leq 1.0$. Hence the amount of fractional coexistence stays higher and the drop in fractional coexistence in $c_c \times p_s$ parameter space is more precipitous at higher values of t_h that the resolution of figure 5.15 suggests.

Two more simulations were carried out to look at the effects of changing the horizontal transmission rate, t_h, on population dynamics in the stable region of $c_c \times p_s$ parameter space. Suitable values for the parameters c_c and p_s were determined through plots made using the method in section 4.4.9 again. To allow for comparison with previous results, the default parameters from the Kwiatkowski and Vorburger (2012) study were again used, but in these simulations $c_c = 0.5$, $p_s = 0.75$, $c_i = 0$ and $t_h = 0.001$ with $t_v = 0.95$ and $t_v = 0.9999$. The resulting population dynamics are shown in figure 5.17. It can be seen that increasing the vertical transmission rate here increases the final size of the infected host population but drives the uninfected host population to less than 1 individual, which can be reasonably interpreted as biologically extinct. The number of individuals entering the partial refuge created by the protective secondary endosymbiont was 0.233 when $t_v = 0.95$ and 0.0005197 when $t_v = 0.999$, the corresponding number of individuals leaving the partial refuge were 48.11 and 0.1045 respectively. In both cases, the magnitude of the number of individuals leaving the refuge was higher than the number of individuals entering the refuge, but the group of individuals with the biggest magnitude was the number of individuals leaving the partial refuge when $t_v = 0.95$. The effect of this is to maintain a larger pool of uninfected hosts when $t_v = 0.95$ than when $t_v = 0.9999$.

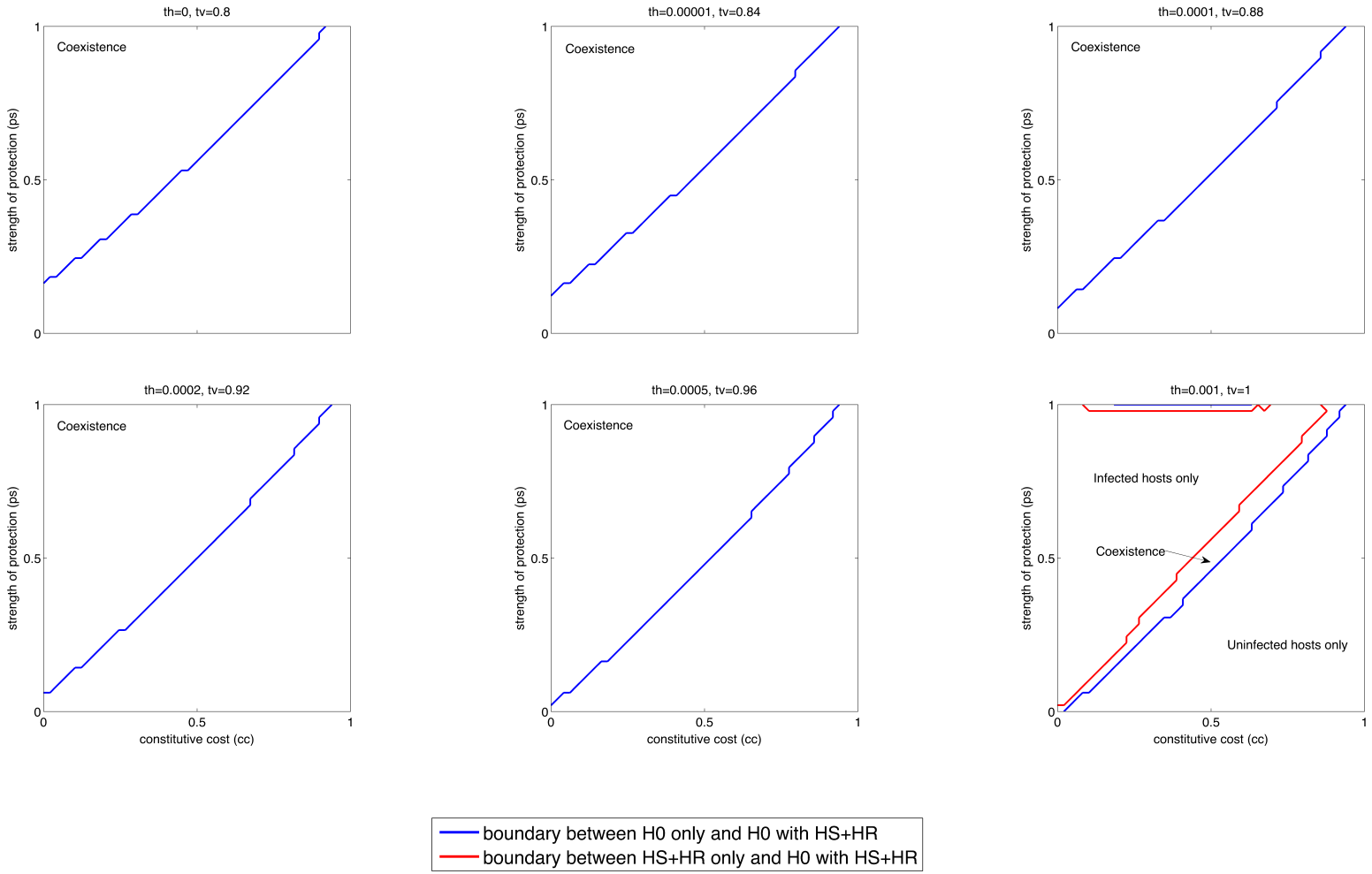


Figure 5.16: Result of simulations to determine the boundaries between persistence of the uninfected host population, H_0, only or the infected host population, $H_S + H_R$, only and coexistence of the uninfected and infected host populations, $H_S + H_R$, in the $c_c \times p_s$ parameter space for differing horizontal and vertical transmission rates.

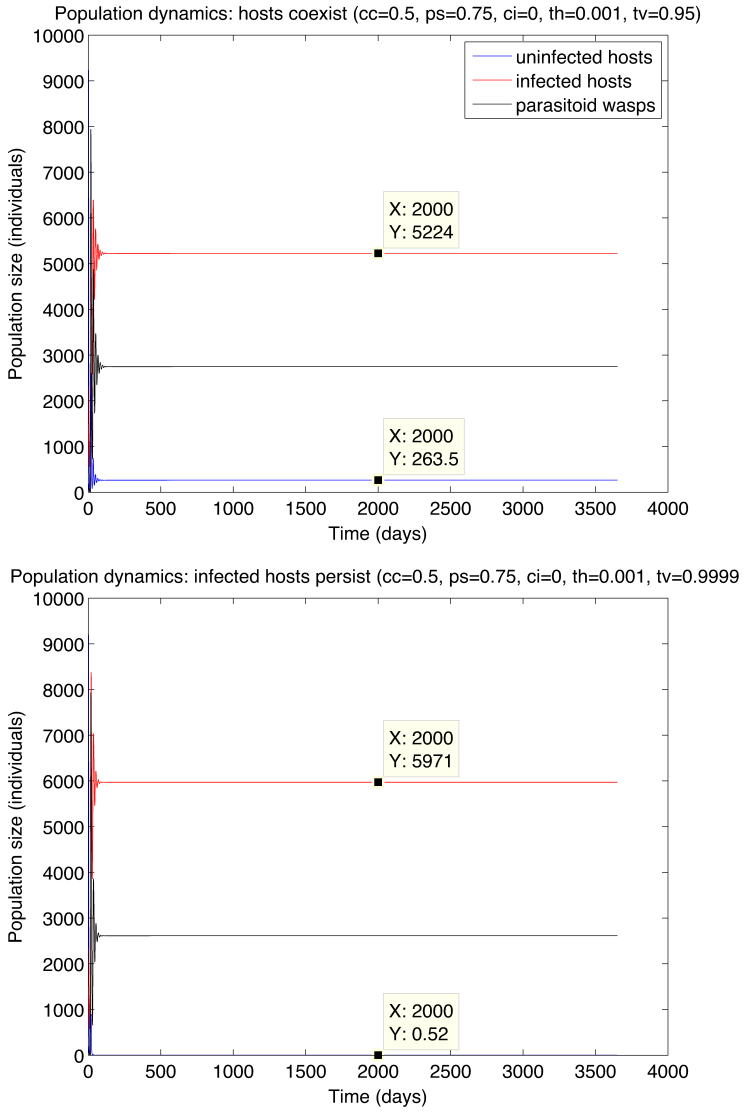


Figure 5.17: Results of two simulations exploring the effect of changing horizontal transmission rate, t_h, on the size of final populations in the region of stable population dynamics.

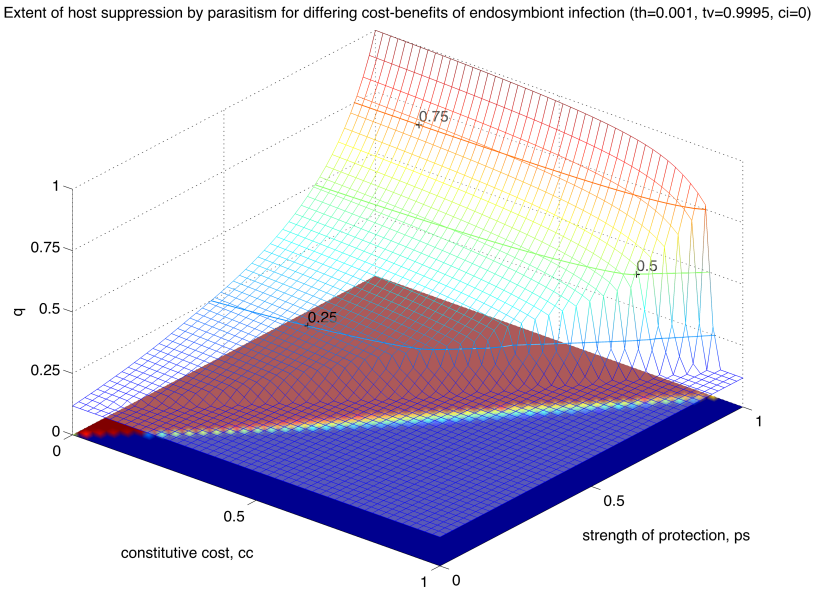


Figure 5.18: Plot of q overlaid on parameter sweep showing suppression of host populations by parasitoid.

5.2.4 Suppression of host populations below carrying capacity by predation

Section 4.4.10 describes the parameter, $q = \frac{\text{mean } H_0 + \text{mean } H_S + \text{mean } H_R}{k}$, that can give an indication of the effectiveness of the parasitoid wasps as natural enemies in this biological system. Two sets of simulations were carried out using the new model to explore the extent of this suppression of host populations by predation, q, in the region of stable final population outcomes as endosymbiont transmission rates were varied. The effect of such suppression is shown in figure 5.18. When q is higher, the degree of suppression of host populations is lower. This plot overlays a sweep across $c_c \times p_s$ parameter space with a 3 dimensional plot of the value of q for each point in the sweep. Contours showing the values $q = 0.25$, $q = 0.5$ and $q = 0.75$ are displayed. Figure 5.19 shows the lower level of figure 5.18 with the boundary between stable and oscillating populations overlaid on the sweep in black.

Two sets of simulations were carried out using the new model to explore the extent of this suppression of host populations by predation, q, in the region of stable final population outcomes as endosymbiont transmission rates were varied.

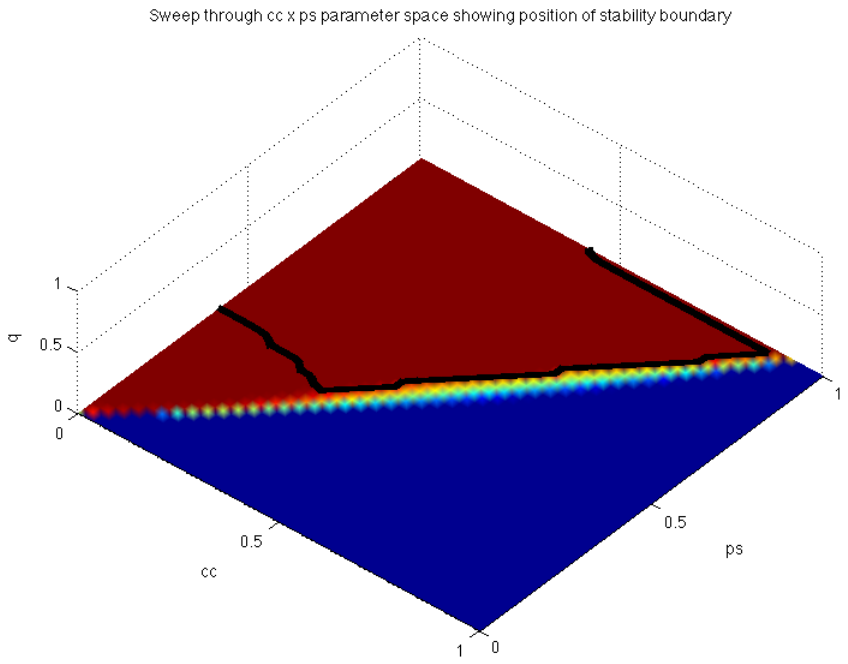


Figure 5.19: Lower level of q plot showing parameter sweep with stability boundary marked.

The boundaries between stable and oscillatory final population outcomes were determined as described in section 4.4.9 and indicated in figure 5.19. In addition, the extent of host population suppression, q, was calculated for each grid point. The resulting plots (figure 5.20 and 5.21) combine this information to show the range of values of host population suppression below carrying capacity that the parameter q may take, depending on the strength of protection afforded by the secondary endosymbiont infection (not shown), as the constitutive cost changes. The plots further show the size of the stable region for this set of parameter values.

The first set of simulations (figure 5.20) looked at the effect of changing the vertical (maternal) transmission rate, t_v, on the extent of suppression of host populations, q. The second set of simulations (figure 5.21) looked at the effect of changing the horizontal (conspecific) transmission rate, t_h, on the suppression of host populations by predation, q, for the maximum and minimum vertical transmission rates, $t_v = 1.0$ and $t_v = 0.8$ respectively. The induced cost of harbouring a secondary endosymbiont infection was held at zero throughout these simulations. The lower middle plot in figure 5.20 is the closest in parameter values to the values

used in the simulation resulting in figures 5.18 and 5.19, and the shape and extent of the stable region can be seen to be consistent between both representations.

When the rate of vertical transmission is increased from $t_v = 0.8$ to $t_v = 1.0$, the size of the region of stable final population outcomes increases, and, for low to midrange values of the constitutive cost, c_c, the range of q increases from approximately $2.4 \leq q \leq 0.9$ to approximately $0.19 \leq q \leq 1$. When q increases, then the degree of suppression decreases and *vice versa*. The change in the upper limit of q means that the parasitoid wasps are less effective at controlling hosts population sizes for the corresponding low cost but high benefit endosymbiont infection parameter values.

When the rate of horizontal transmission is increased from $t_h = 0.0$ to $t_h = 0.001$, there is no noticeable change, within the computational limits of the model, in the size of the region of final population stability. Further, there is near negligible change in the suppression of the host populations by predation. As these simulations were repeated for the maximum and minimum values of the rate of vertical transmission, $t_v = 1.0$ and $t_v = 0.8$ respectively, it is reasonable to assert that the same trend would be observed for the similar simulations carried out for intermediate values of t_v. Changes along the lower boundary are due to the highly oscillatory dynamics in the region that cause values to fluctuate widely depending on whether the data point recorded corresponds to a peak or trough in the oscillation.

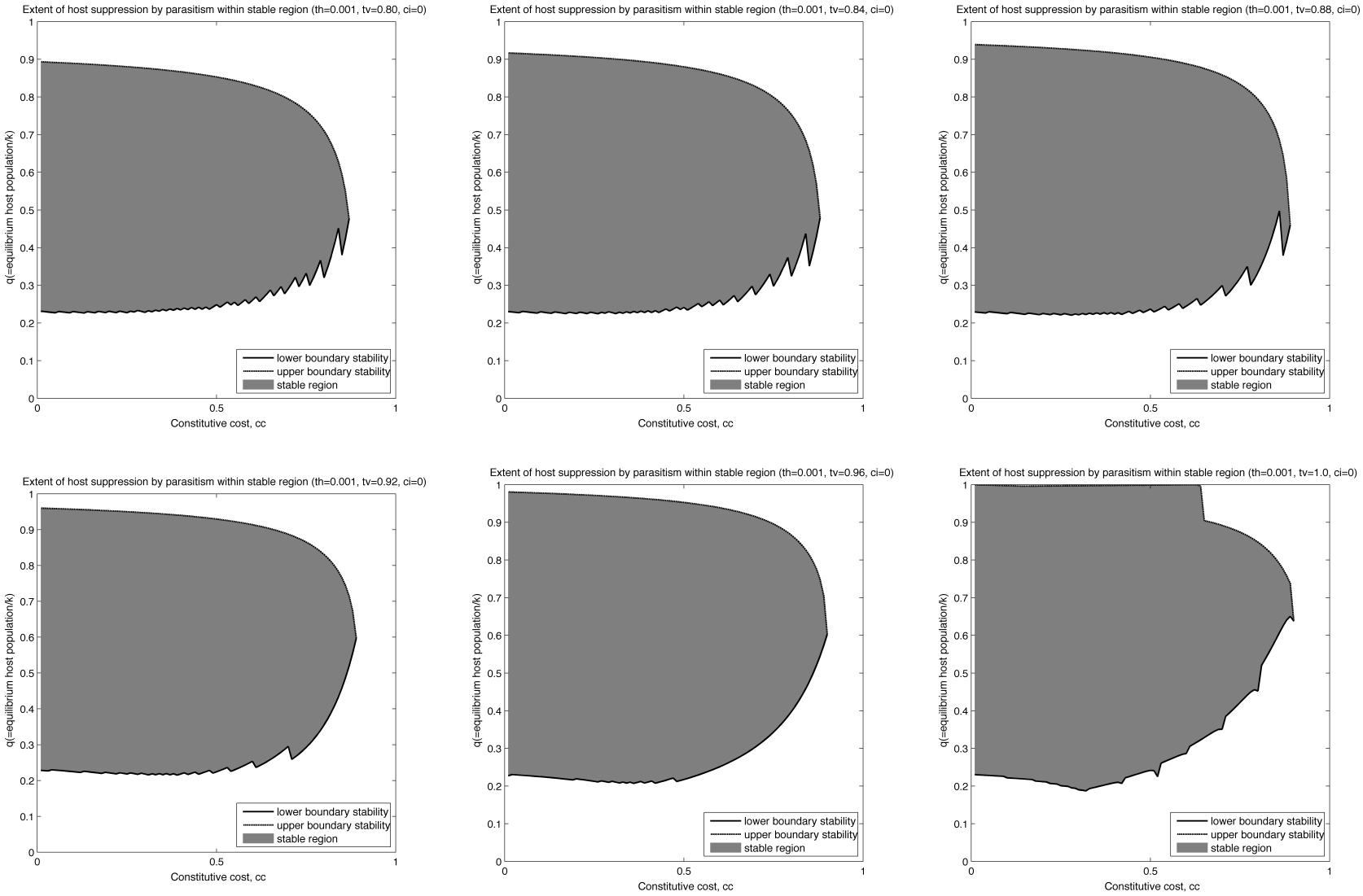


Figure 5.20: Results of simulations exploring the effect of changing vertical transmission rate, t_v, on the suppression of host populations by predation, q.

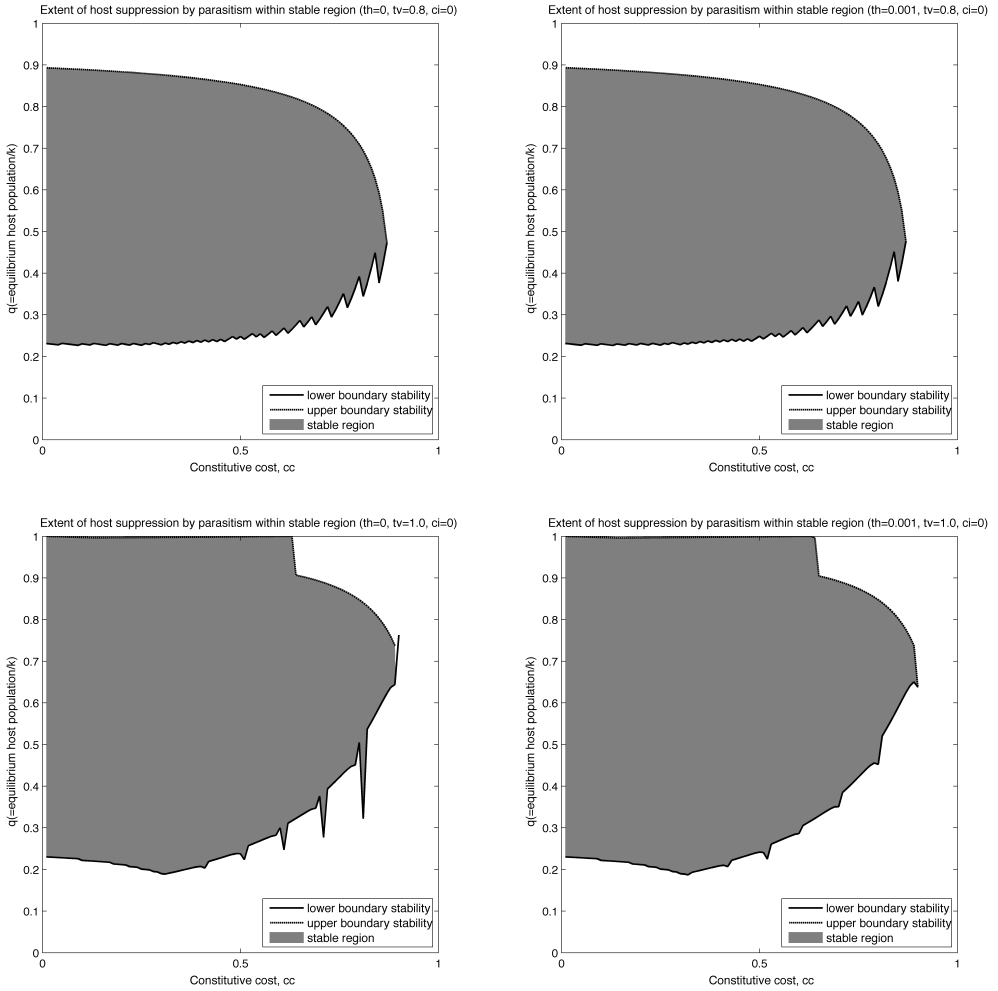


Figure 5.21: Results of simulations exploring the effect of changing horizontal transmission rate, t_h, on the suppression of host populations by predation, q for the maximum (below) and minimum (above) vertical transmission rates, t_v.

5.2.5 Results from simulations using pea aphid parameter values

Three types of simulation were run again with the new model code and with parameters taking mid-range values from the set of pea aphid parameter values compiled in table 4.2. The simulations were:

1. sweeps through $c_c \times p_s$ parameter space for increasing induced costs of harbouring a secondary endosymbiont infection,

2. identification of the boundaries between stable and oscillatory final population outcomes, and also between persistence of the uninfected host population, H_0, only and coexistence of the uninfected and infected host populations, $H_S + H_R$, in the $c_c \times p_s$ parameter space for increasing induced costs of harbouring a secondary endosymbiont infection,

3. sweeps across $c_c \times p_s$ parameter space, each overlaid with a 3 dimensional plot of the value of q for each point in the sweep, for increasing induced costs of harbouring a secondary endosymbiont infection.

The mid-range parameter values used were $b_h = 2.8$, $d_h = 0.0375$, $d_p = 0.136$, $t_h = 0.0005$ and $t_v = 0.9$. The induced costs simulated ranged from $c_i = 0$ (no cost) to $c_i = 0.969$ (32-fold cost).

The results from the sweeps through $c_c \times p_s$ parameter space for increasing induced costs of harbouring a secondary endosymbiont infection and identification of the boundaries between stable and oscillatory final population outcomes, and also between persistence of the uninfected host population, H_0, only and coexistence of the uninfected and infected host populations, $H_S + H_R$, in the $c_c \times p_s$ parameter space for increasing induced costs of harbouring a secondary endosymbiont infection (figures 5.22 to 5.27) show the same trends as the simulations carried out in section 5.2.1.1. As the induced cost of harbouring a secondary endosymbiont infection, c_i, increases, the range of cost-benefit values promoting coexistence decreases from $c_c = 0.0, p_s = 0.08$ to $c_c = 0.94, p_s = 1.0$ when $c_i = 0$, and from $c_c = 0.0, p_s = 0.88$ to $c_c = 0.35, p_s = 1.0$ when $c_i = 969$, and the region of uninfected host dominance (shown by the dark blue areas in these figures) increases. Further, as the induced cost of harbouring a secondary endosymbiont infection, c_i, increases, the range of cost-benefit values promoting stability decreases from $c_c = 0.0, p_s = 0.39$ to $c_c = 0.90, p_s = 1.0$ when $c_i = 0$, and from $c_c = 0.0, p_s = 0.92$ to $c_c = 0.43, p_s = 1.0$ when $c_i = 875$. When there is a very

high (32-fold) induced cost, none of the populations are stable. The degree of suppression of host populations by parasitism ranges from $0.15 \leq q \leq 0.96$ when $c_i = 0$ and $0.15 \leq q \leq 0.40$ when $c_i = 0.969$.

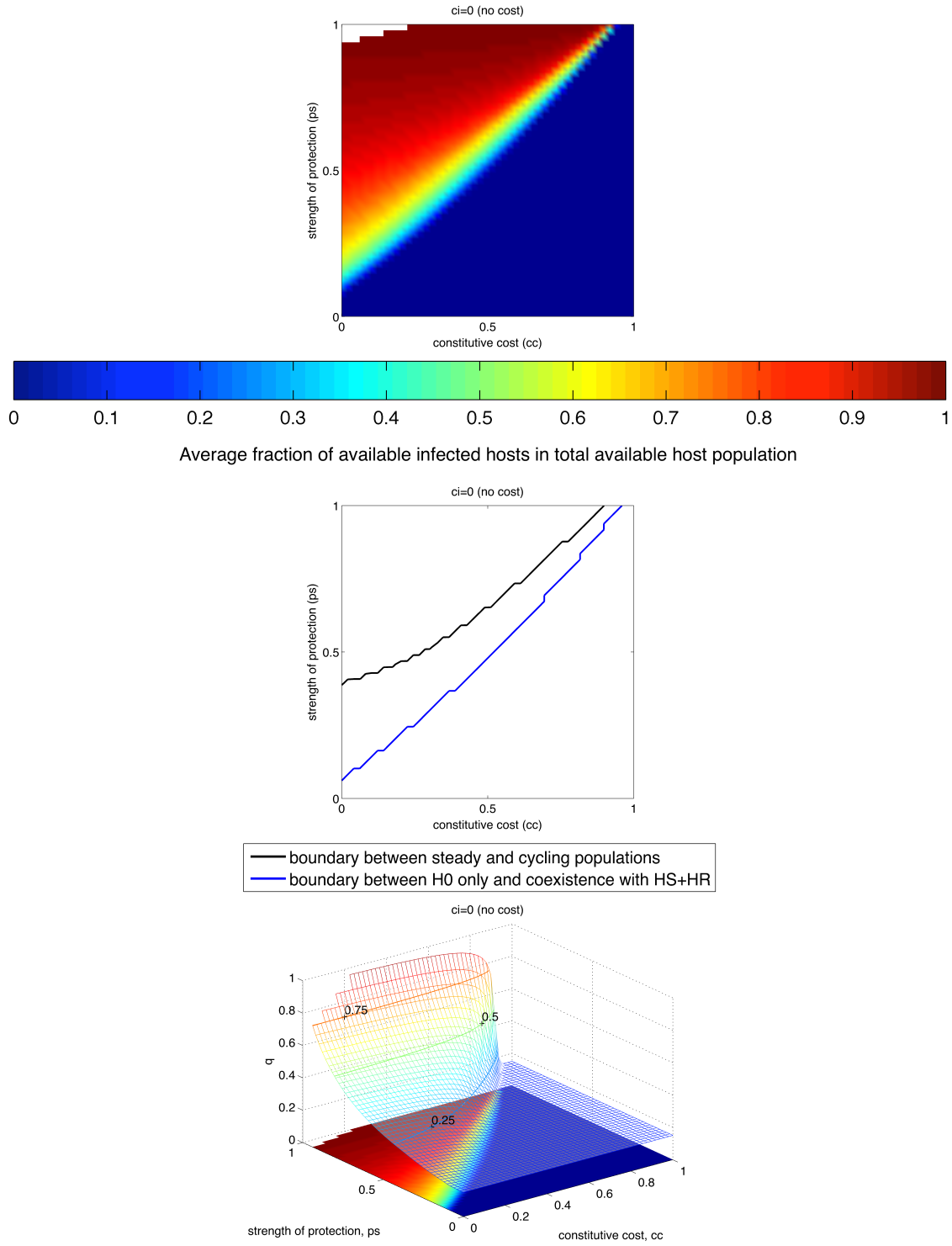


Figure 5.22: Results of simulations 1 (top), 2 (middle) and 3 (bottom) as listed in section 5.2.5 when there is no induced cost of harbouring a protective secondary endosymbiont infection. White area in top figure indicates total population extinction.

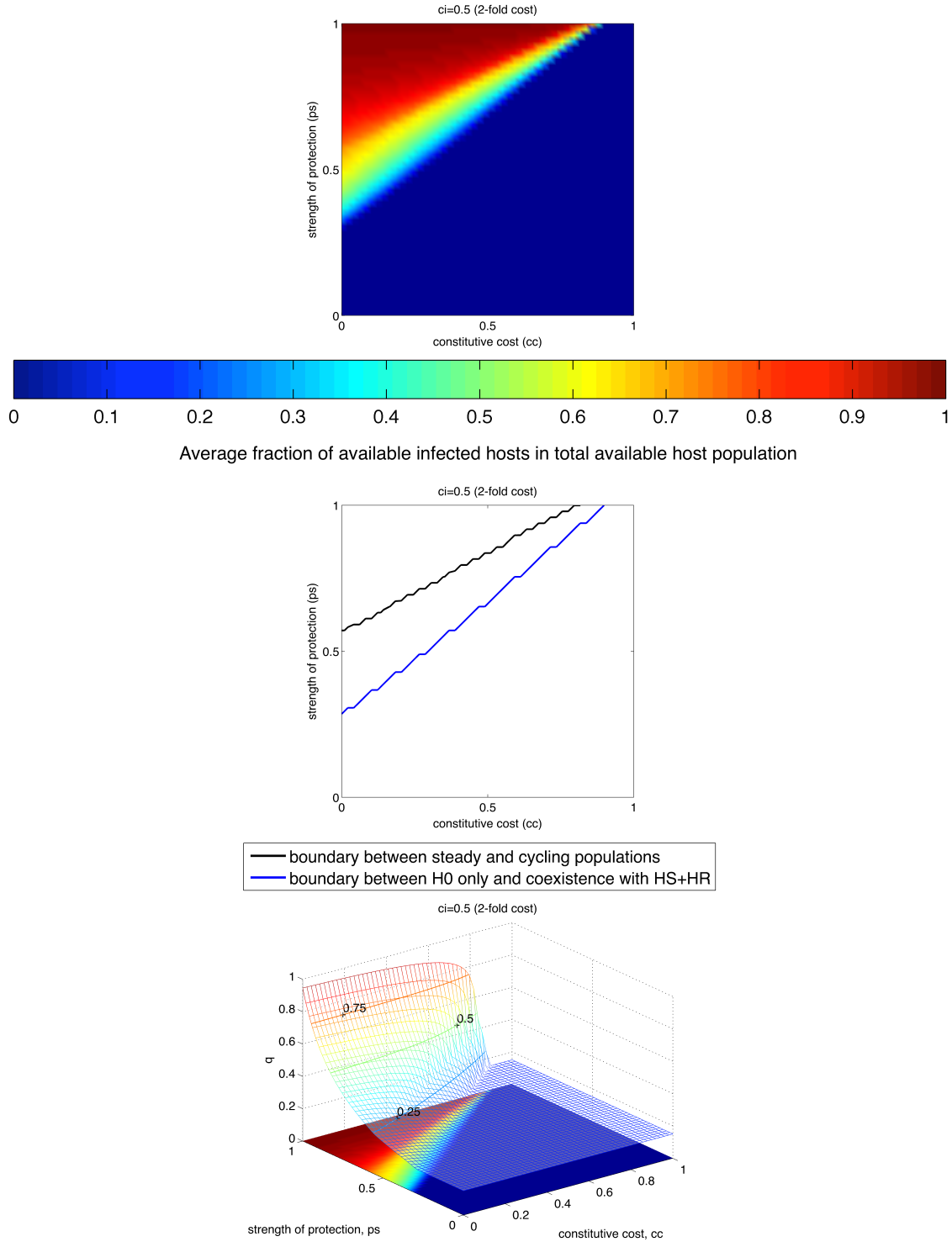


Figure 5.23: Results of simulations 1 (top), 2 (middle) and 3 (bottom) as listed in section 5.2.5 when there is a 2-fold induced cost of harbouring a protective secondary endosymbiont infection.

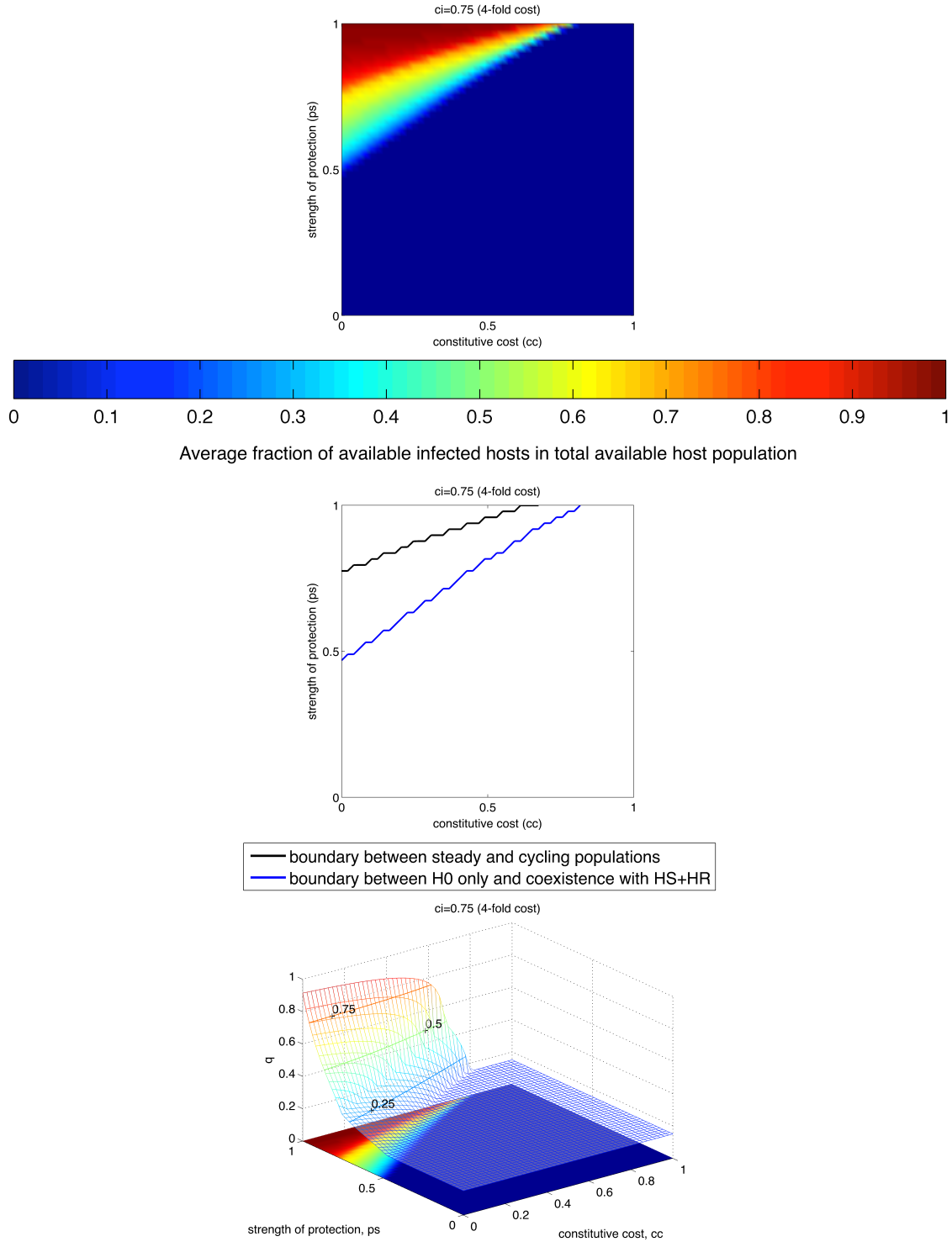


Figure 5.24: Results of simulations 1 (top), 2 (middle) and 3 (bottom) as listed in section 5.2.5 when there is a 4-fold induced cost of harbouring a protective secondary endosymbiont infection.

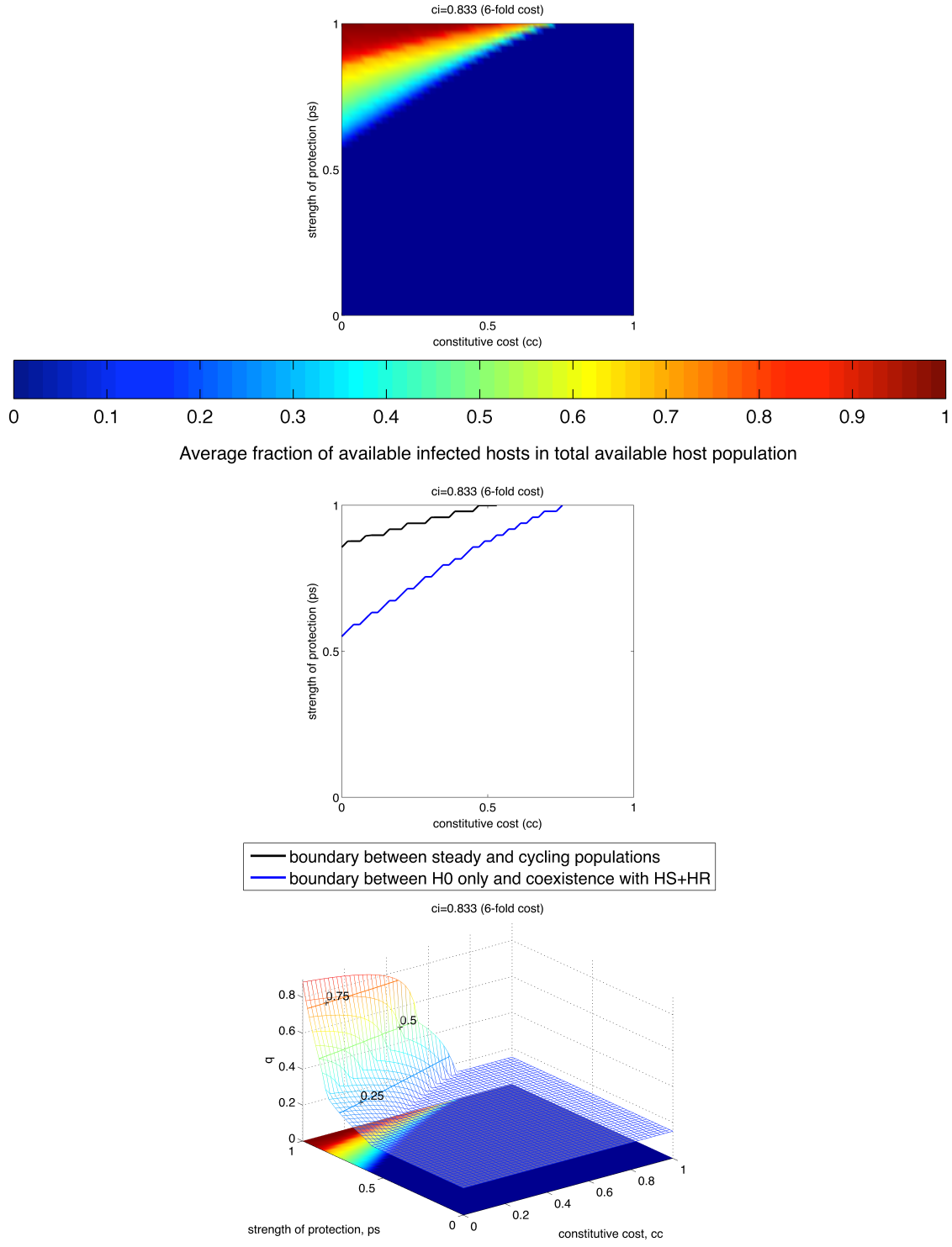


Figure 5.25: Results of simulations 1 (top), 2 (middle) and 3 (bottom) as listed in section 5.2.5 when there is a 6-fold cost of harbouring a protective secondary endosymbiont infection.

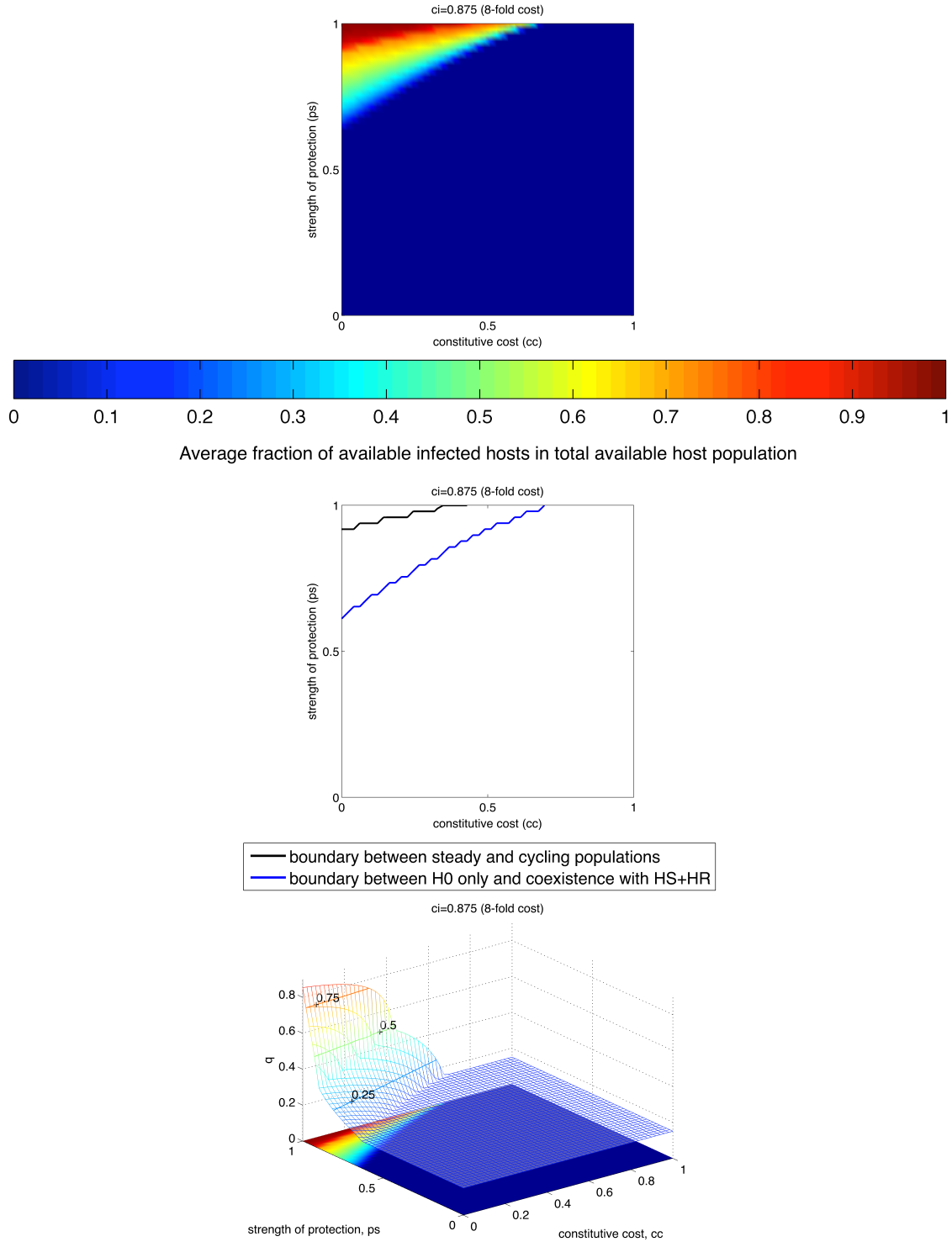


Figure 5.26: Results of simulations 1 (top), 2 (middle) and 3 (bottom) as listed in section 5.2.5 when there is an 8-fold induced cost of harbouring a protective secondary endosymbiont infection.

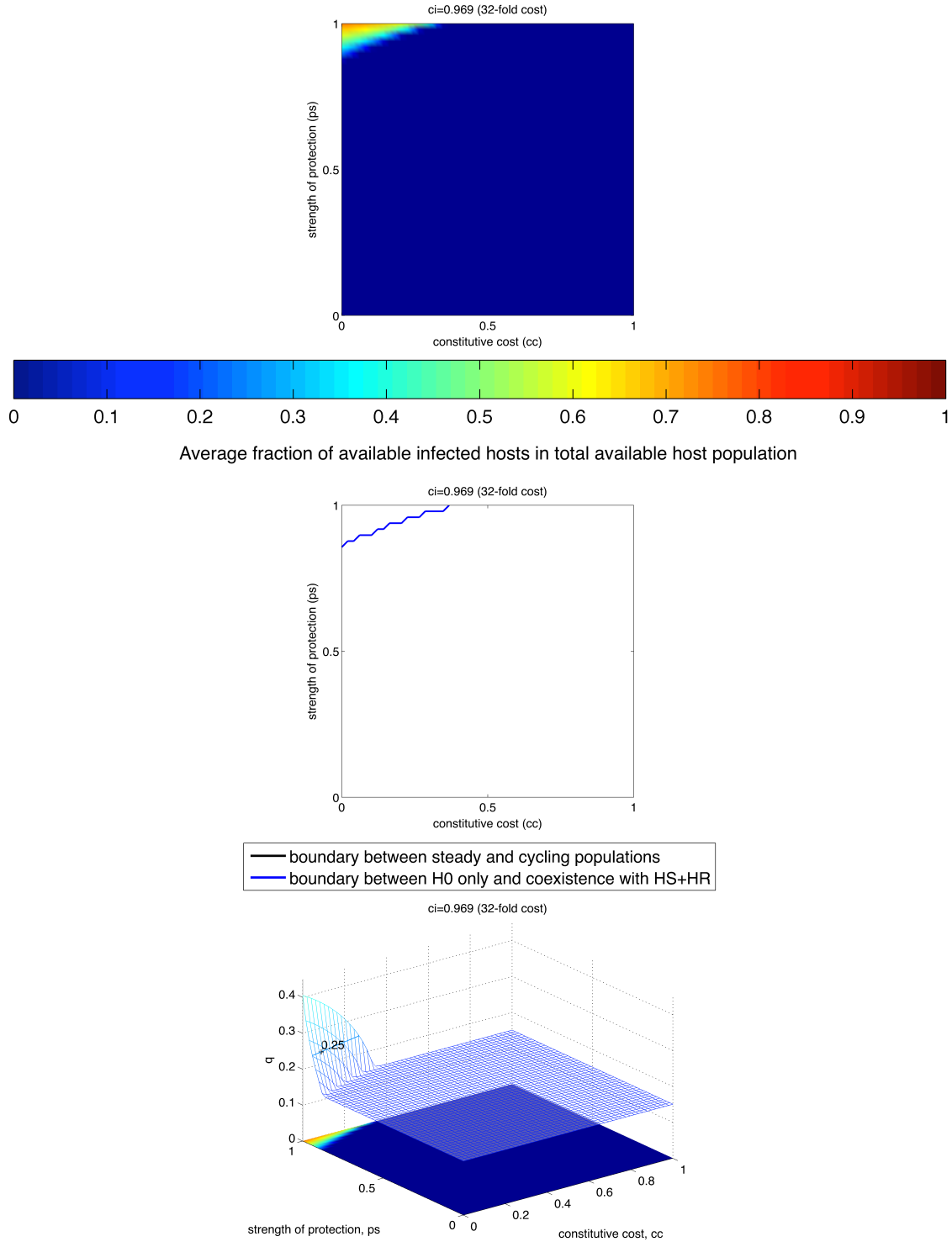


Figure 5.27: Results of simulations 1 (top), 2 (middle) and 3 (bottom) as listed in section 5.2.5 when there is a 32-fold cost of harbouring a protective secondary endosymbiont infection.

5.2.6 Strength of protection provided by endosymbiont to pea aphids on second and subsequent attacks by parasitoid wasps

All simulations carried out so far have assumed that the strength of protection provided to the aphid host by the secondary endosymbiont infection has remained constant on second and subsequent attack by parasitoid wasps. Figure 5.28 shows the effect on the fractional amount of coexistence, as the horizontal and vertical transmission rates are varied, when the strength of protection afforded by the secondary endosymbiont is halved and lost (relative to the strength of protection in figure 5.15) respectively. In these two simulations, the parameter took the default values from Kwiatkowski and Vorburger (2012), except that p_{sp} took the values $\frac{1}{2} \times p_s$ and $0 \times p_s$ respectively. Reducing the amount of protection given by the secondary endosymbiont on second and subsequent attack lowered the fractional amount of coexistence. Oliver et al. (2012) report that the *H. defensa* secondary endosymbiont infection is ineffective against superparasitism and, overall, may even enhance parasitoid wasp success if such *H. defensa* infected aphids are preferentially targeted instead of uninfected aphids.

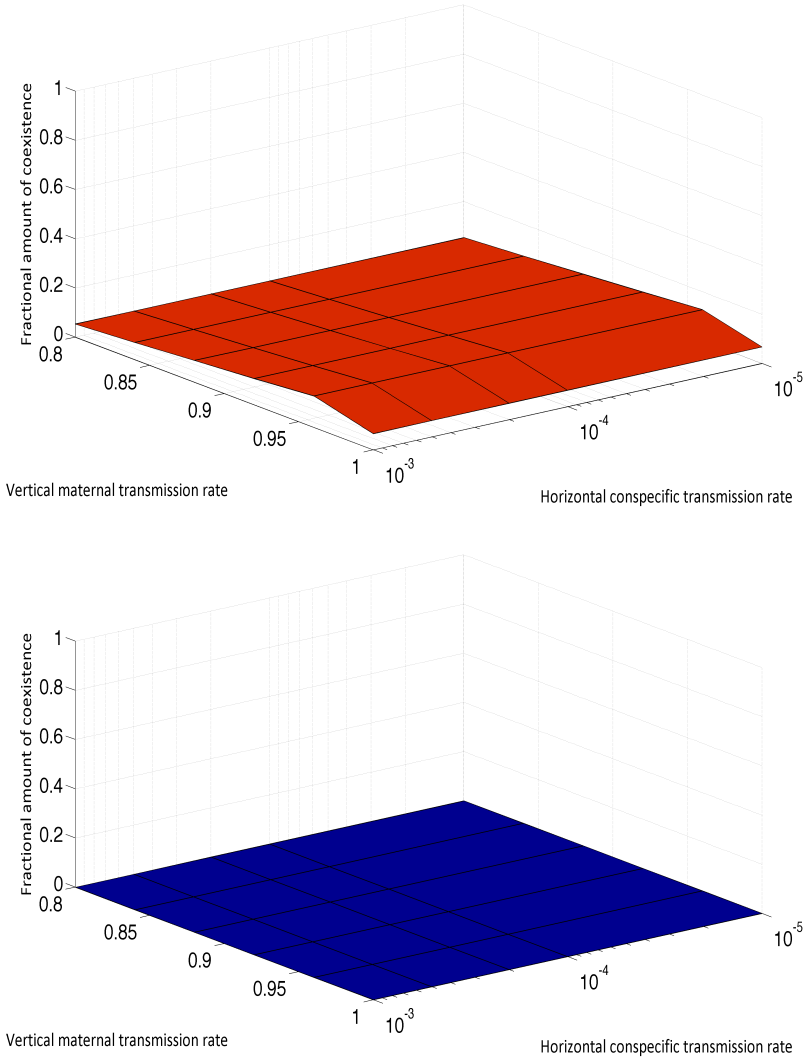


Figure 5.28: Endosymbiont protection halved on second and subsequent attacks and endosymbiont protection lost on second and subsequent attacks.

Table 5.1: Parasitoid choice: fractions of each host population class attacked

Host class:	Fraction attacked per trial:		Mean:
Uninfected hosts	0.745	0.819	0.782
Infected hosts attacked once	0.189	0.232	0.211
Attacked infected hosts attacked again	0.611	0.636	0.624

5.2.7 Parasitoid choice

So far, it has been assumed that parasitoids do not discriminate between uninfected and infected hosts. There has been an absence of selective pressure in the model as it has been purely deterministic in nature. Oliver et al. (2012) carried out behavioural arrays to investigate if *A. ervi* tend to selectively parasitise *H. defensa* infected pea aphids. Their results suggest oviposition may not be random, but that parasitoid oviposition decisions may be informed by host infection status. Whilst it would be possible to modify the mathematical model to incorporate a non-random parasitoid search with an aggregated response, a rough indication of the effect of such choice can be introduced into the model using the data generated by Oliver et al. (2012).

The quantity "fraction attacked" in the model was replaced by fractions of uninfected and infected hosts taken from data from Oliver et al. (2012) summarised in table 5.1 and the mean values calculated. The mean total fraction of uninfected hosts with 1 or more eggs present upon dissection was used to inform the fraction of uninfected hosts attacked. The mean fraction of *H. defensa* aphids with 1 egg present was taken as a measure of the fraction of infected hosts attacked once, whilst the mean fraction of *H. defensa* aphids with more than 1 egg present was taken as a measure of the fraction of infected attacked hosts subsequently attacked again.

The three types of simulation run in section 5.2.5 were repeated, modifying the MATLAB code with these fixed values for the fractions attacked. The induced costs again ranged from $c_i = 0$ (no cost) to $c_i = 0.969$ (32-fold cost). The mid-range parameter values used were, again, $b_h = 2.8$, $d_h = 0.0375$, $d_p = 0.136$, $t_h = 0.0005$ and $t_v = 0.9$.

The results from the sweeps through $c_c \times p_s$ parameter space for increasing in-

duced costs of harbouring a secondary endosymbiont infection and identification of the boundaries between stable and oscillatory final population outcomes, and between persistence of the uninfected host population, H_0, only, and coexistence of the uninfected and infected host populations, $H_S + H_R$, in the $c_c \times p_s$ parameter space for increasing induced costs of harbouring a secondary endosymbiont infection (figures 5.29 to 5.31) show the same general trends as the simulations carried out in sections 5.2.1.1 and 5.2.5. As the induced cost of harbouring a secondary endosymbiont infection, c_i, increases, the range of cost-benefit values promoting coexistence and stability decreases, but less markedly, and the region of uninfected host dominance (shown by the dark blue areas in these figures) increases. Further, as the induced cost of harbouring a secondary endosymbiont infection, c_i, increases, the range of cost-benefit values promoting stability decreases. The shape of the boundaries differs from those resulting from previous simulation with the boundaries approaching the vertical as the induced cost increases. Even when the induced cost is very high (32-fold), a region of stable coexistence exists for all $0.0 \leq p_s \leq 1.0$ when $c_c \leq 0.27$. The degree of suppression of host populations by parasitism ranges from $0.20 \leq q \leq 0.96$ when $c_i = 0$ and $0.20 \leq q \leq 0.86$ when $c_i = 0.969$.

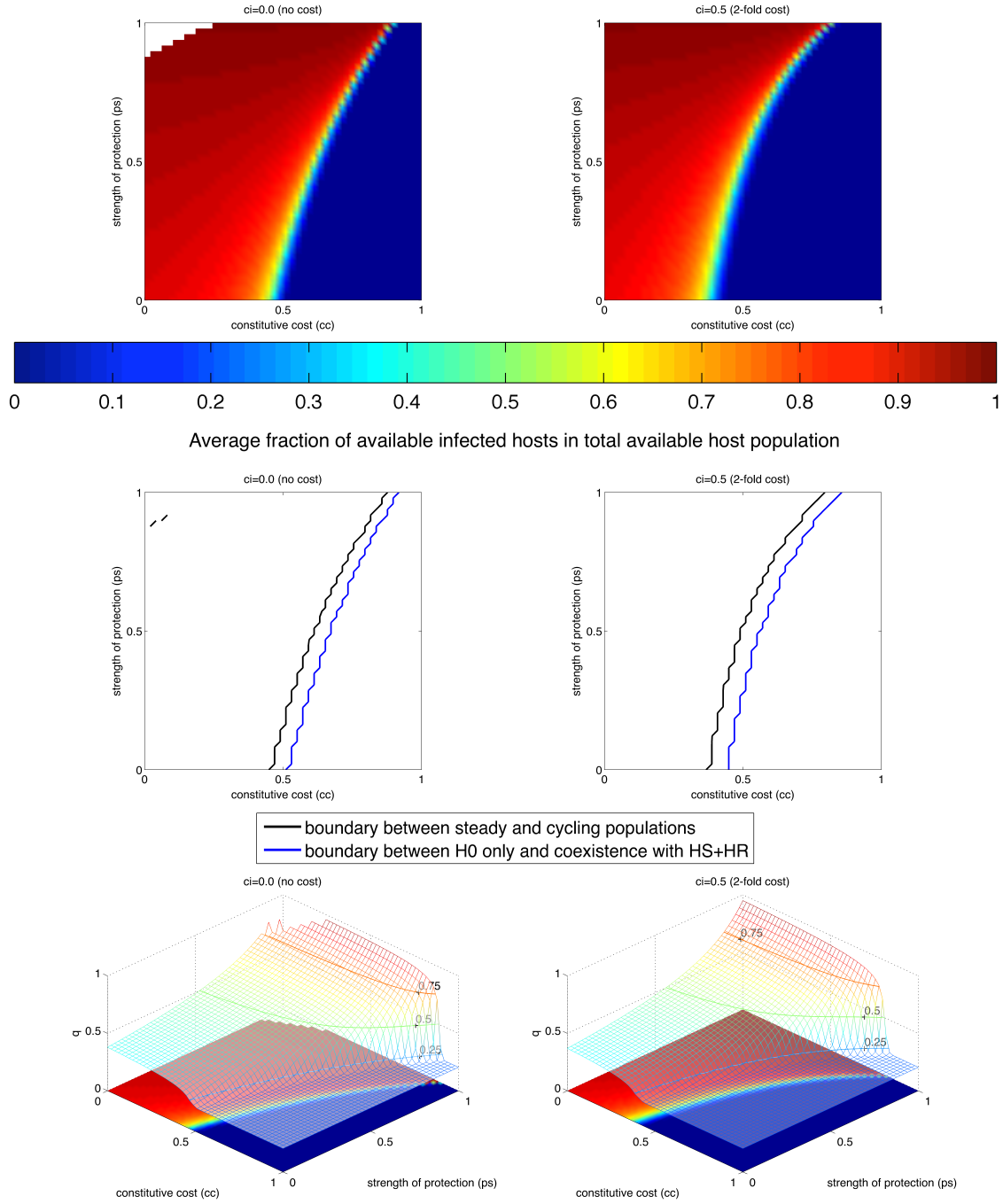


Figure 5.29: Results of simulations 1 (top), 2 (middle) and 3 (bottom) as listed in section 5.2.5 when there is no induced cost (left) and a 2-fold induced cost (right) of harbouring a protective secondary endosymbiont infection. White area in top figure indicates total population extinction.

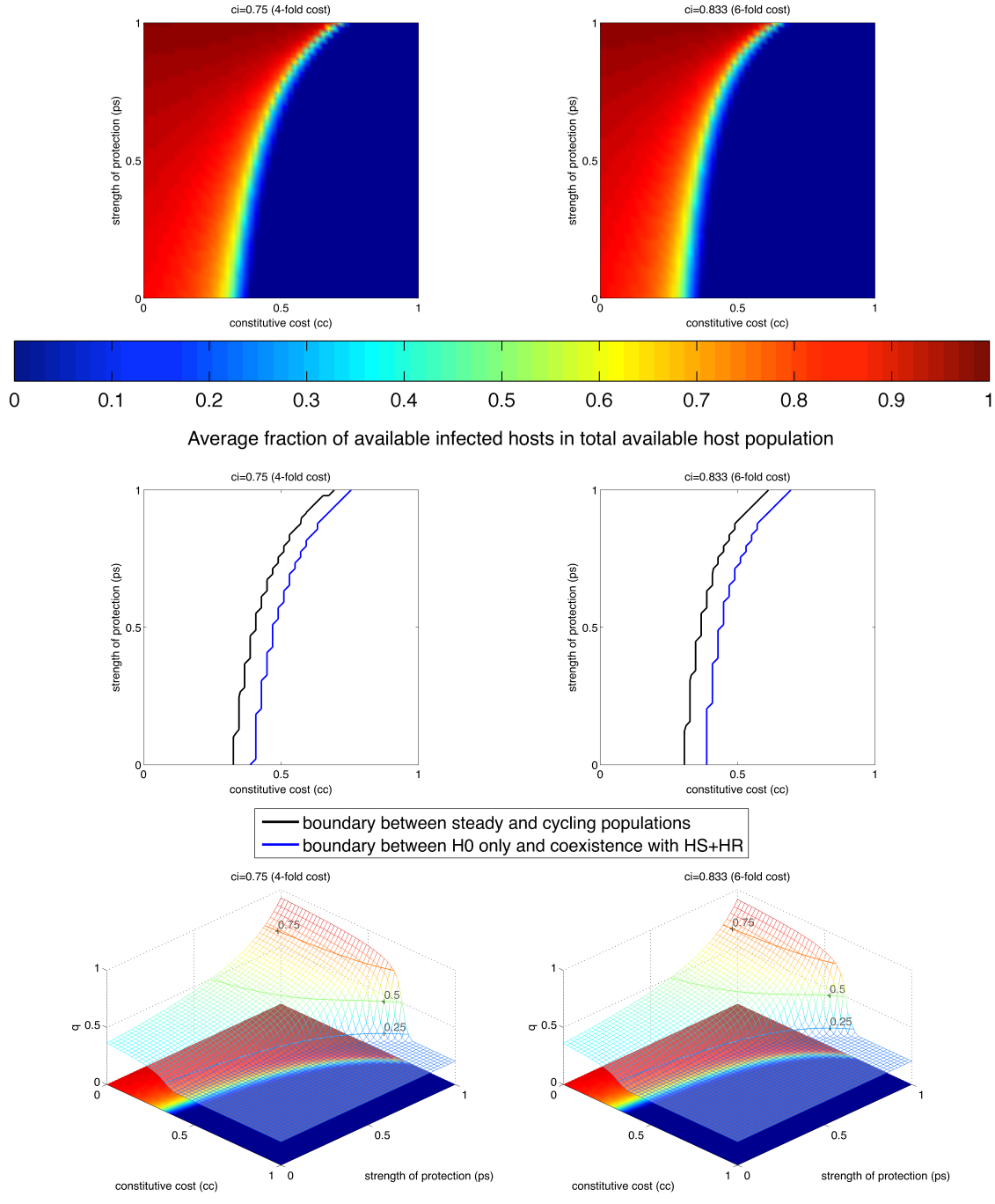


Figure 5.30: Results of simulations 1 (top), 2 (middle) and 3 (bottom) as listed in section 5.2.5 when there is a 4-fold induced cost (left) and a 6-fold induced cost (right) of harbouring a protective secondary endosymbiont infection.

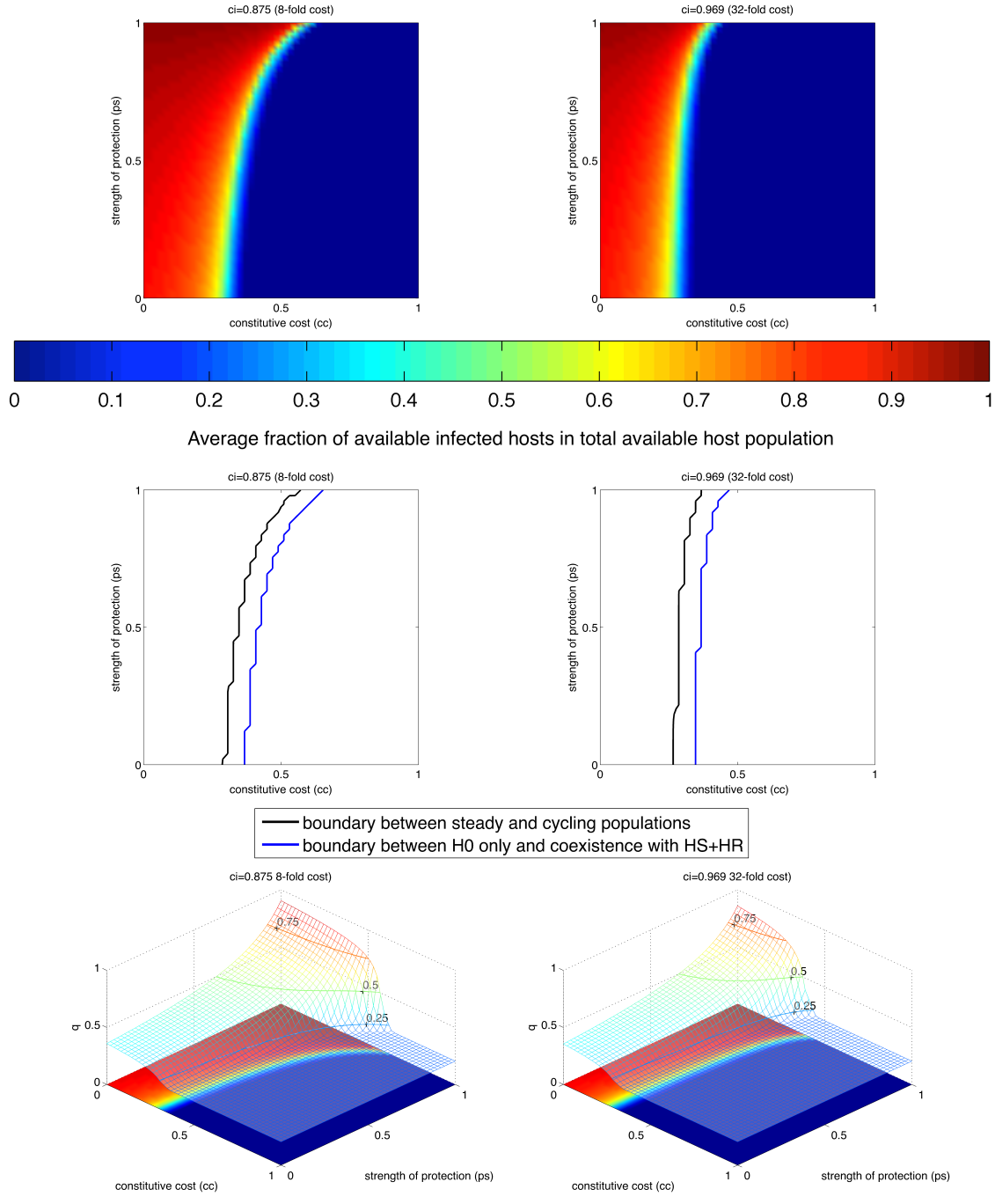
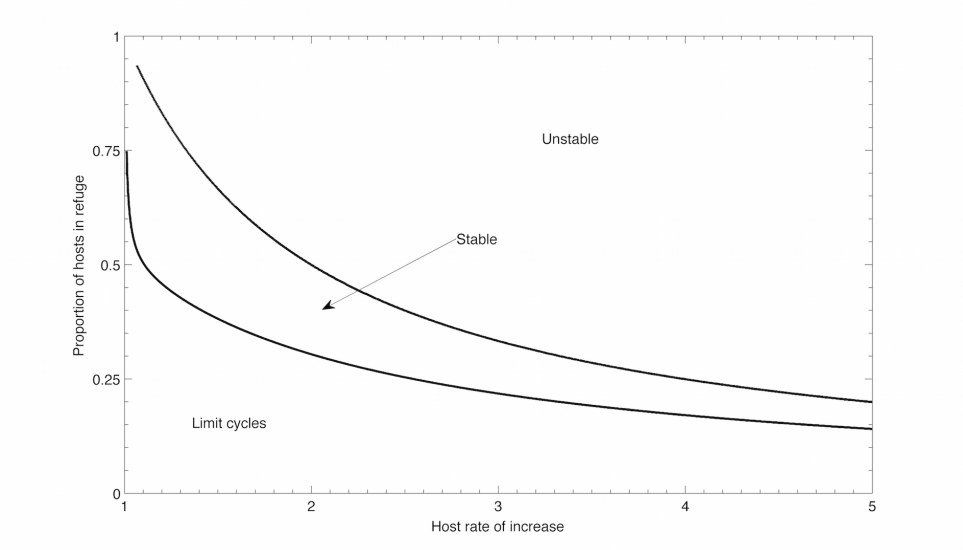


Figure 5.31: Results of simulations 1 (top), 2 (middle) and 3 (bottom) as listed in section 5.2.5 when there is an 8-fold induced cost (left) and a 32-fold induced cost (right) of harbouring a protective secondary endosymbiont infection.

Figure 5.32: Reproducing stability boundaries from Godfray and Hassell (1991).



5.2.8 Effect of "all-or-none" encapsulation

5.2.8.1 Encapsulation and population stability

After writing code in MATLAB to simulate the population dynamics described by equations (1.8) used by Godfray and Hassell (1991) to explore the effects of encapsulation on population stability as the rate of host population growth increases, the computational method developed to find the stability boundaries described in section 4.4.9 was used to verify the stability properties of the Godfray and Hassell (1991) model with a type I functional response $f\left(H(t), P(t)\right) = \exp\left(-aP(t)\right)$. The results of this simulation are shown in figure 5.32, and these results broadly replicate the results obtained by Godfray and Hassell (1991). In the absence of an explicit value in the Godfray and Hassell (1991) paper, the search efficiency, a, was taken to be $a = 0.068$ for this simulation. This parameter value was determined experimentally by Burnett (1958) using greenhouse whitefly, *T. vaporariorum*, and its parasitoid *E. formosa*.

"Populus" is software containing a set of population biology and evolutionary ecology simulations. For the default set of "Populus" parameters ($\lambda = 2$, $a = 0.068$, $H = 25$, $P = 10$ and $\eta = 0$), the basic Nicholson-Bailey model results generated by the MATLAB code were checked against the output of simulations carried out in "Populus" for the same mathematical model (figure 5.33), and identical plots showing unstable host and parasitoid population size oscillations leading to extinc-

tion were obtained for this parameter set. Both variations in host and parasitoid population sizes with time and phase-plane host-parasitoid dynamics are plotted.

Following verification of the model, a further simulation (figure 5.34) was carried out after incorporating a type II functional response into the basic encapsulation model proposed by Godfray and Hassell (1991) with parameters specific to pea aphids. In this case $a = 146$ and $b = 0.0011$ (Snyder and Ives, 2003). The value of $\lambda \approx 6$ for the pea aphid host population was estimated from the pea aphid birth and death rate where $\lambda = b_h - d_h$. This value was slightly higher than any measured value used in this study, but allows for exploration of a possible higher population growth rate. The range reduced from full population growth rate to no population growth as if a constitutive reproductive cost had been incurred as in the model proposed by Kwiatkowski and Vorburger (2012). The variation in encapsulation response is analogous to either a variation in innate resistance to parasitism in uninfected hosts, or to variation in overall resistance (innate and endosymbiont conferred) in an infected aphid. The proportion of hosts in a partial refuge is here, therefore, due to either of these cases. Stability boundaries were determined computationally as before, and phase-plane plots of N against P (not shown) generated to confirm the type of dynamics in each region of the graph.

The resulting dynamics are limit cycles or extinction when the proportion of the pea aphid population in the partial refuge is approximately 25% or lower throughout the full population growth rate range, and also when the proportion in a refuge increases to over 75% as the population growth rate drops below 2. The region of stable population outcomes is narrower in the results of this pea aphid specific simulation compared to figure 5.32 and final population sizes are smaller. At very low rates of population increase, stability is a rare outcome. For other parameter values, the population dynamics are unstable.

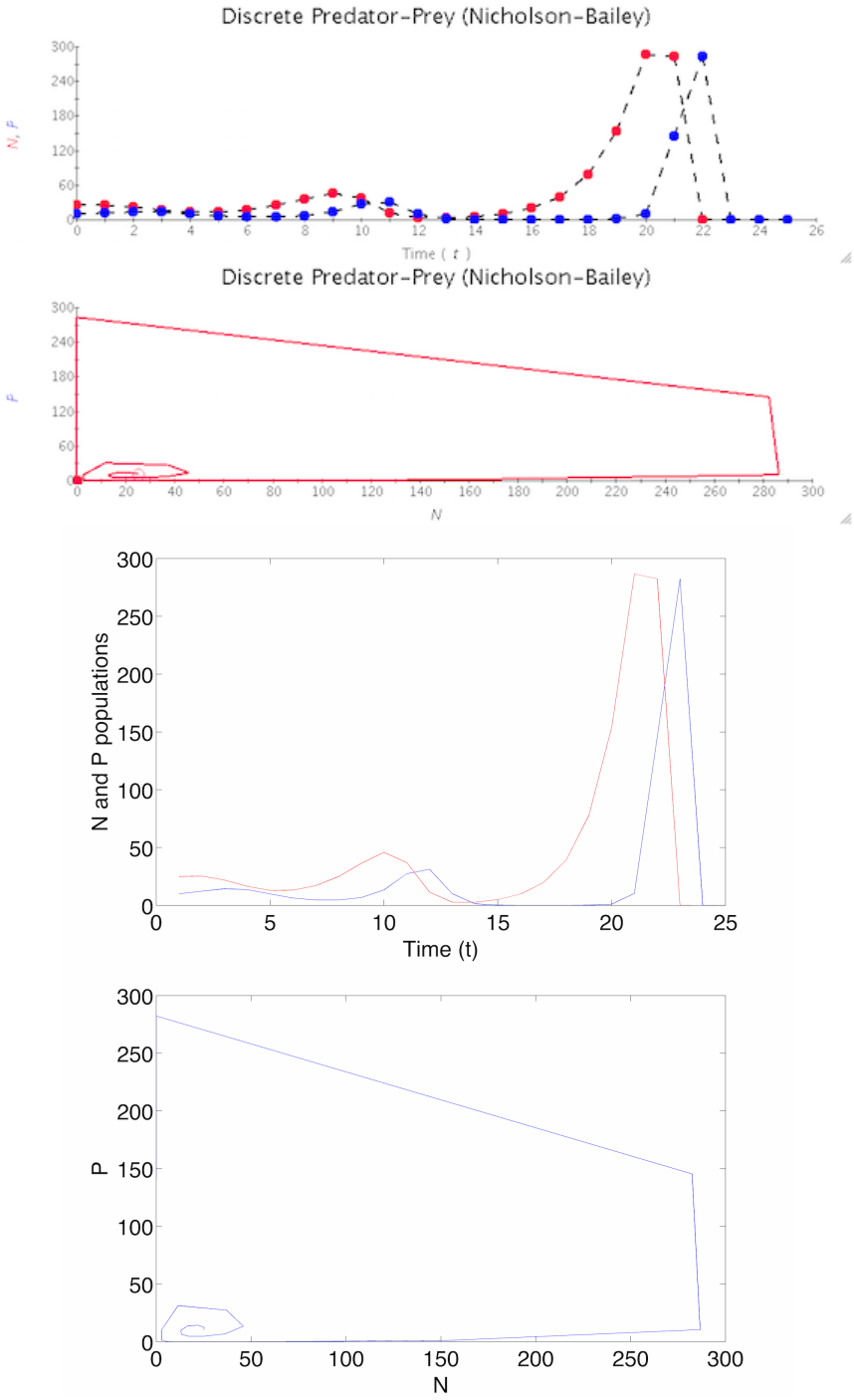
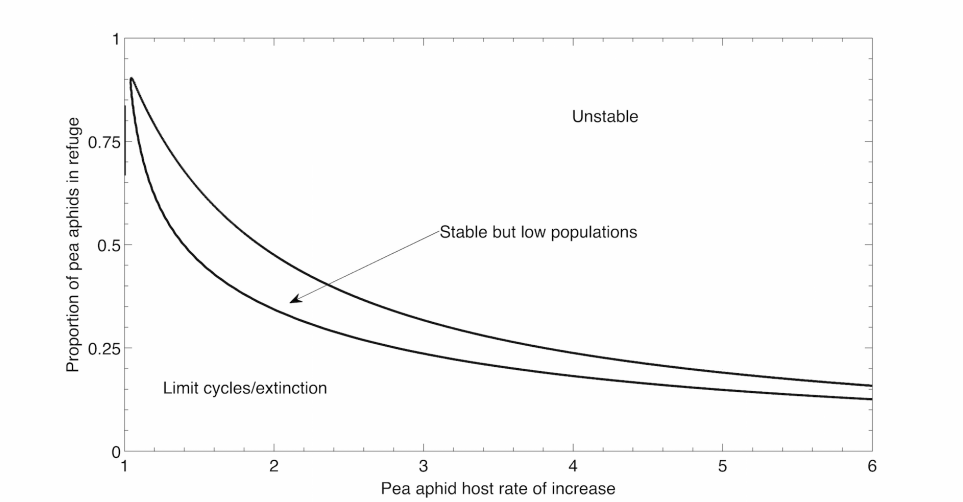


Figure 5.33: Populus output with $\eta = 0$ (top) and MATLAB output with $\eta = 0$ bottom.

Figure 5.34: Modified Godfray and Hassell (1991) model with pea aphid parameters and a type II functional response.



5.2.8.2 Evolution of encapsulation with resource limitation

The model proposed by Godfray and Hassell (1991) (described by equations (1.10)) to investigate the evolution of encapsulation with resource limitation was coded in MATLAB. Again, a type I functional response of the form $f\left(H(t), P(t)\right) = \exp\left(-aP(t)\right)$ was used. A simulation was carried out using a search efficiency of $a = 0.068$ and other parameters taking fixed values from their simulation such that $K = 1000$, $\lambda_1 = 4.5$, $\eta_1 = 0.3$, whilst the outcome was explored over the parameter ranges for the "mutant" strain $1 \leq \lambda_2 \leq 6$ and $0 \leq \eta_2 \leq 1$. The outcome of this simulation were plotted as a grayscale pseudo colour plots of the final populations sizes for varying encapsulation ability and fecundity (figure 5.35).

In the plot of the size of the original resident population, N against "mutant" fecundity and encapsulation ability, the white area of the plot corresponds to displacement of the "resident" population by the "mutant" population. The results are similar to those of Godfray and Hassell (1991). The original "resident" host population is displaced as the fecundity and encapsulation ability of the "mutant" population increase. Corresponding plots for S ("mutant" population) and P (parasite population) are included. The "mutant" population peaks for high "mutant" fecundity and encapsulation ability. The wasp population peaks for at high "mutant" fecundity and mid-range "mutant" encapsulation ability.

The parameter values corresponding to coexistence of original and "mutant"

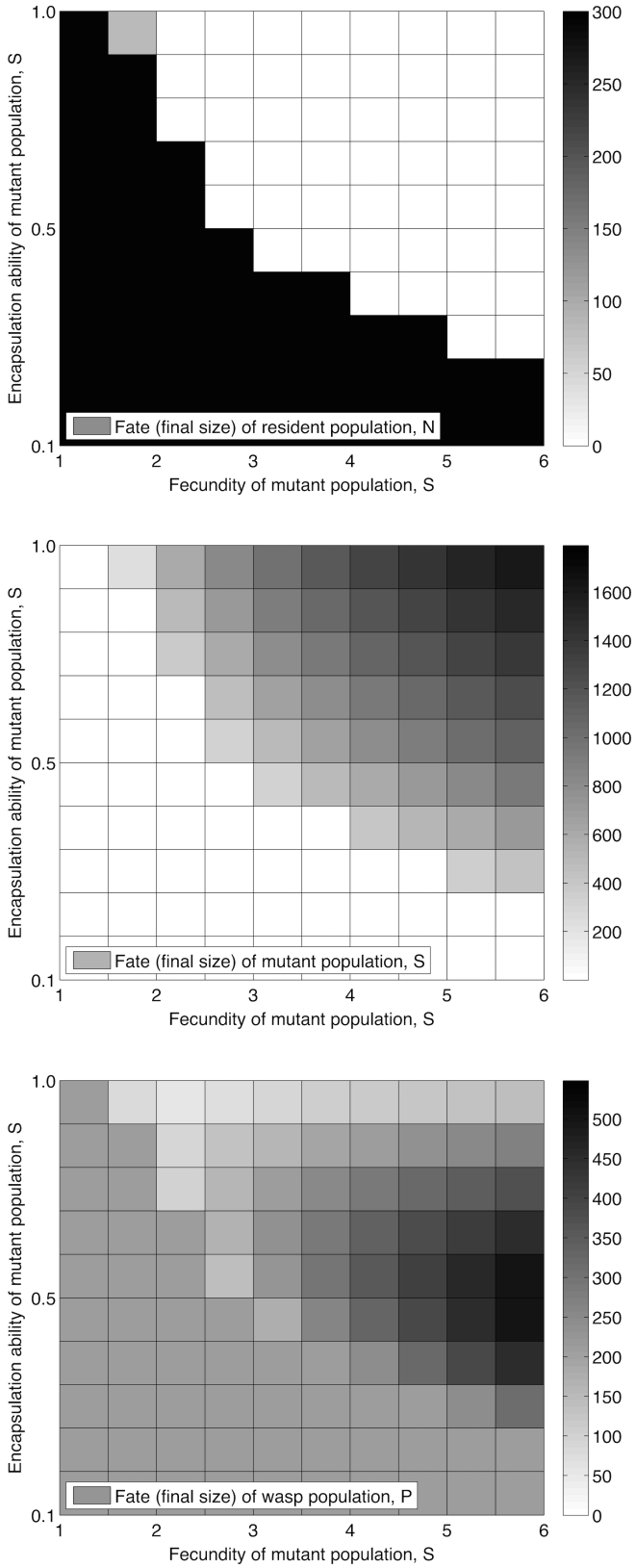
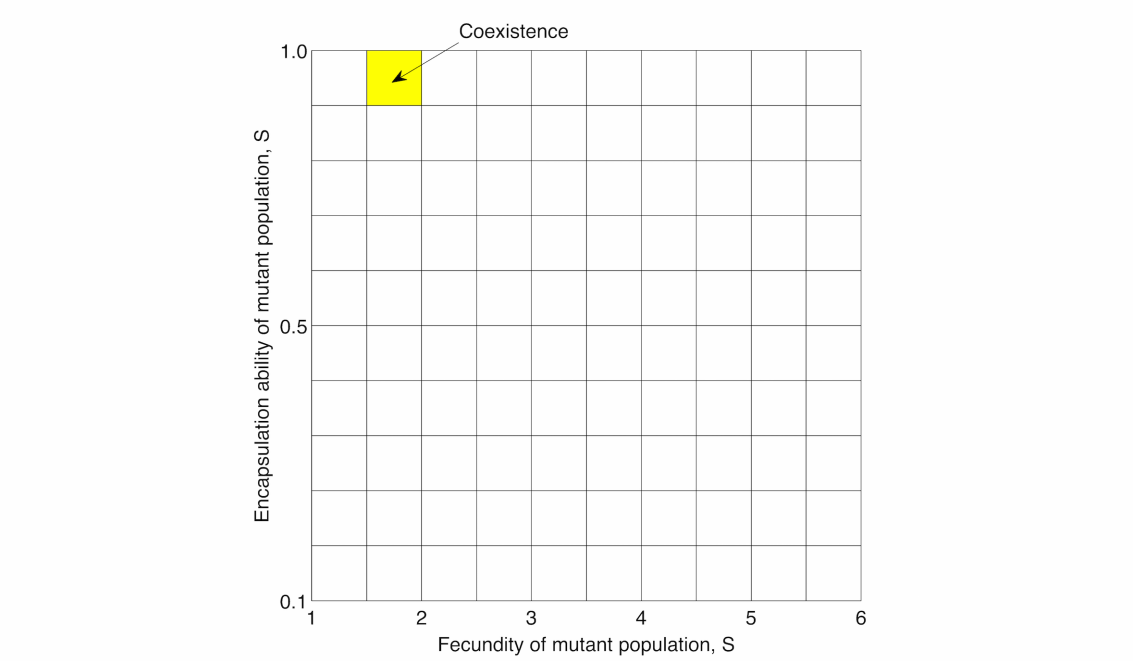


Figure 5.35: Final host and parasitoid population sizes from the Godfray and Hassell (1991) model.

Figure 5.36: Coexistent final host and parasitoid populations from Godfray and Hassell (1991) model.



host populations were identified and plotted as the yellow area in figure 5.36. Coexistence is a rare outcome, existing here only when the fecundity of the "mutant" population is just less than 2 and the encapsulation ability of the "mutant" population is nearly 1.

For the next simulation, the resolution was increased from dividing the sweep area into a 10×10 meshgrid to a 100×100 meshgrid, and the colormap was changed to the same as used for the pseudocolor plots arising from Kwiatkowski and Vorburger (2012) described in section 4.4.8. The blue area indicates that the original population had not become extinct, the red region indicates where the original population had become extinct. The results of a sweep through $\lambda \times \eta$ parameter space with parameter values kept the same as in section 5.2.8 is shown in the top plot in figure 5.37. Generating another plot of the same data but with the x axis expressed in terms of a *cost* to the fecundity of the "mutant" population, S, yields the bottom plot in figure 5.37. In essence, this figure is a sweep through $c_c \times p_s$ parameter space with the fate of each population expressed through the final population size in absolute terms, rather than as a fraction of infected hosts. Note that this model has no induced cost of harbouring an endosymbiont, no

transmission of endosymbionts, and no time delays but does include an element of host population density-dependence. The constitutive cost quantity from the Kwiatkowski and Vorburger (2012) model c_c is akin to the cost the fecundity of the "mutant" host population, whilst the Kwiatkowski and Vorburger (2012) strength of protection quantity p_s is akin to the encapsulation ability.

The final set of simulations repeat the simulations carried out to generate figures 5.35 and 5.36 but using a much higher resolution, the same colormap as the Kwiatkowski and Vorburger (2012) sweeps, parameter values drawn from the set of pea aphid parameter values (section 4.4.3) and the x axis expressed in terms of a fecundity cost to the "mutant" population, S. The results are shown in figures 5.38 and 5.39. Figure 5.38 shows the fates of the original ("uninfected") population, "mutant" ("infected") population and parasitoid population. The original population dominates from the region of low cost, low encapsulation ability through to the region of high cost, high encapsulation ability. "Mutant" hosts dominate in the region of low to medium costs with medium to high encapsulation ability. The parasitoid population peaks in size in the region of low cost and medium encapsulation ability. Figure 5.39 shows coexistence between original and "mutant" strains of aphid host. The region of coexistence is small and lies along the boundary between dominance of each host type.

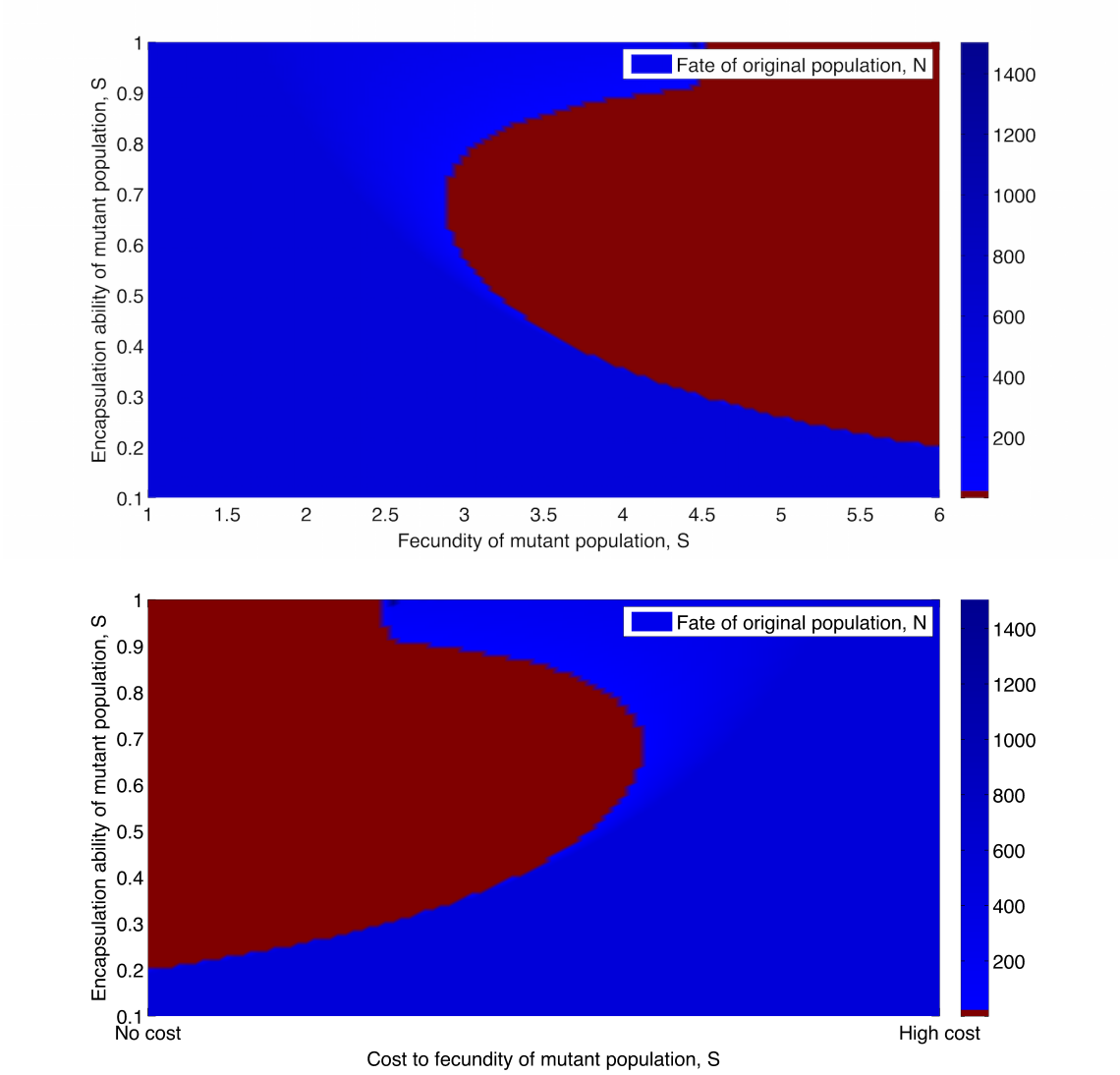


Figure 5.37: Fate of original population when invaded by "mutant" population following Godfray and Hassell (1991) (top), and fate of original population when invaded by "mutant" population following Godfray and Hassell (1991) in terms of cost to fecundity of "mutant" population, S (bottom).

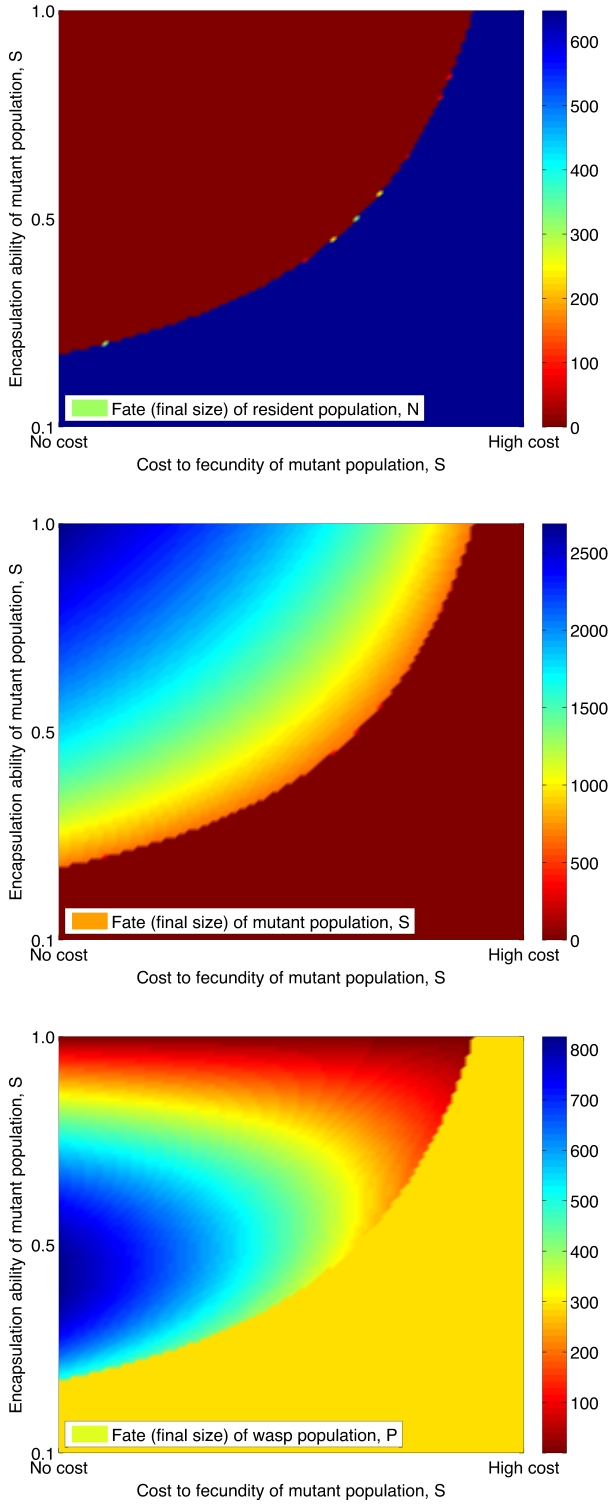
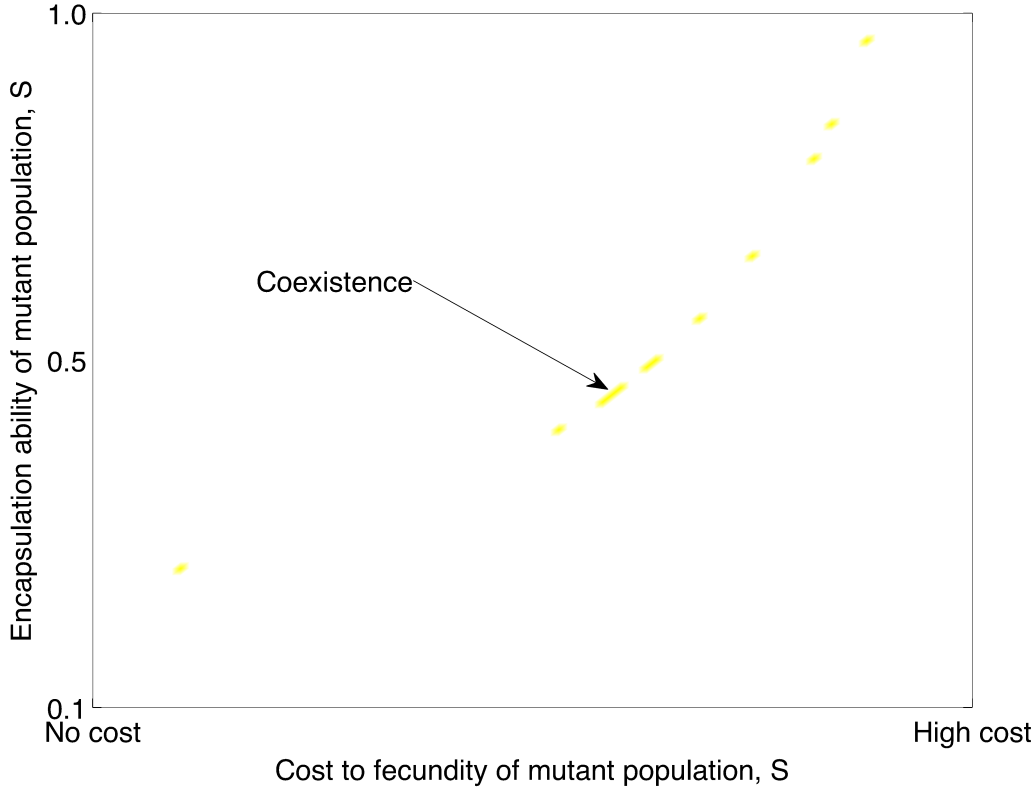


Figure 5.38: Fate of original population when invaded by mutant population following Godfray and Hassell (1991) (top), and fate of original population when invaded by mutant population following Godfray and Hassell (1991) in terms of cost to fecundity of mutant population, S (bottom). Model parameterised using pea aphid data.

Figure 5.39: Coexistent final host and parasitoid populations from Godfray and Hassell model when parameterised using pea aphid data



5.3 Discussion

The key findings of the study by Kwiatkowski and Vorburger (2012) were that their simulations showed that coexistence between uninfected and infected aphid hosts was precarious, occurring only in the simplest of cases for a very limited range of cost and benefit parameter combinations. The authors of the study found that increasing the induced cost of harbouring a secondary endosymbiont infection widened the range of cost and benefit parameter values required for host coexistence. The authors also concluded that high vertical and low horizontal endosymbiont transmission rates resulted in more observed cases of coexistence, but noted that 100% fidelity of maternal transmission was an exception to this trend. Finally, the authors postulated that populations outcomes (extinction or otherwise) were "coupled" to the population dynamics. In particular, the authors state that "population levels stabilize if and only if unprotected hosts become extinct".

Comparing the results of this study to the findings of Kwiatkowski and Vorburger (2012), the key findings here are:

1. Coexistence between uninfected and infected aphid hosts was a relatively common population outcome for the simplest case of the model when the constitutive cost was, approximately, in the lower half of its range and the beneficial strength of endosymbiont protection was in the upper half of its range. It should be noted that persistent coexistent uninfected host populations were small. However, it should also be noted that the model developed in this study did not incorporate any form of selective pressure on populations. The introduction of selection would likely affect final population sizes. An indication of the effect of parasitoid attack preference was simulated using experimental data from the published literature, and this resulted in coexistence between host populations for all strengths of protection at low to mid range constitutive costs. Hence, selective pressures may promote coexistence in some circumstances.

2. When parasitoids attacked randomly, increasing the induced cost of harbouring an endosymbiont infection decreased the range of cost and benefit parameter values required for host coexistence. The region of $c_c \times p_s$ parameter space where coexistence occurred shifted increasingly towards the low cost, high benefit top left quadrant of the parameter space.

3. Having identified that coexistence was not such a rare outcome as suggested by the Kwiatkowski and Vorburger (2012) study, this present work further explored the effect of changing both horizontal and vertical transmission rates sweeping across $t_h \times t_v$ parameter space looking at the fractional amount of coexistence. Varying the horizontal transmission rate does not appear to affect the fractional amount of coexistence for a particular vertical transmission rate; increasing the fidelity of vertical transmission whilst holding the horizontal transmission rate constant slightly increases the fractional amount of coexistence until a sudden drop in coexistence for near perfect maternal transmission of the protective secondary endosymbiont infection resulting in infected hosts prevailing over uninfected hosts. Further, changing horizontal transmission rates has little, if any, effect on stability boundaries unlike increasing vertical transmission rates that shift stability boundaries towards lower strengths of endosymbiont protection.

4. If the endosymbiont conferred protection is reduced or lost on second and subsequent parasitoid attacks, the fractional amount of coexistence decreased when sweeping across $t_h \times t_v$ parameter space.

5. This study disputes the finding that population outcome are "coupled" (as reported by Kwiatkowski and Vorburger (2012)) to population dynamics, but instead provides evidence from simulations that stable coexistence is a common simulation outcome that does indeed lie within the region of $c_c \times p_s$ parameter space corresponding to estimates from experimental data quoted in the published literature (approximately $c_c <$0.5 and 0.5 $<p_s <$1.0). Unlike the study of Kwiatkowski and Vorburger (2012) that found oscillatory coexistence only, this study finds stable and oscillatory coexistence as population outcomes.

6. The parameter q (Hassell, 1978) describing the degree of suppression of host populations below carrying capacity by predation was introduced as an output from the model in this study. Kwiatkowski and Vorburger (2012) focus mainly on the outcome for host populations but this study allows the amount of depression of host populations by parasitoids in regions of stable host coexistence to be found. This links the costs versus the benefits of an *H. defensa* infection in pea aphids to an assessment of the possible degree of control of pea aphid populations by their natural enemies, *A. ervi* wasps.

After an initial period of irregular oscillations, populations tend towards their final population dynamics and oscillatory dynamics may persist. These oscillations are likely to have been driven in a large part by the time delays in the model expressed as the time to kill an aphid host and the time for a parasitoid wasp to emerge from a dead aphid host. Simulations such as parameter sweeps were not carried out without the time delays present, but it is hypothesised that the population dynamics would be less oscillatory without the time delays. Time delays are often not explicitly modelled in host-parasite dynamics, possibly because of the complexity added to the system. The time delays may also have affected the size of, and the balance between, uninfected and infected final host populations.

Recalling that encapsulation can act as a stabilising factor in host-parasitoid systems, Godfray and Hassell (1991) proffered by way of an explanation that the fraction of hosts invariably safe from predation averts over-exploitation of host populations. Over-exploitation depletes populations faster than they can regenerate. As pea aphid hosts are necessary to complete the parasitoid wasp life-cycle, extinction of the host populations results in extinction of the parasitoid population, such is the intricacy of the host-parasitoid relationship. This explanation is applicable to the results of the modelling work on the cost-benefit trade-offs due to protective *H. defensa* endosymbiont infection in pea aphids.

At the time of publication, Godfray and Hassell (1991) knew of only one study addressing the effect of encapsulation on host-parasitoid dynamics. Rather than using difference equations to model host-parasitoid interactions, that analysis (Hassell and Anderson, 1984) used a Monte Carlo modelling approach comparing a completely susceptible host population (such a population would lack even the innate resistance of the uninfected host population in the Kwiatkowski and Vorburger (2012) model) and a population of varying susceptibility due to immunity analogous to “dosage-dependent” encapsulation rather than “all-or-nothing” encapsulation. Godfray and Hassell (1991) find that the introduction of density-dependence into their model of the evolution of “all-or-none” encapsulation predicts coexistence of “resident” and “mutant” populations for some values of “mutant” fecundity and “mutant” encapsulation ability. There are parallels between the approach of Godfray and Hassell (1991) and Kwiatkowski and Vorburger (2012) and this study applied computational methods developed to explore findings from the modelling work arising from the Kwiatkowski and Vorburger (2012) study to the model presented by Godfray and Hassell (1991) to find stability

boundaries and to mirror the cost versus benefit parameter sweeps in the work of Kwiatkowski and Vorburger (2012) noting broadly similar results from simulations.

Holt and Pickering (1985) discuss the effects of the transmission of infectious disease on community structure using as a basis the SIR model of infection. They highlight that transmission of infection is likely to be important. The model used in this study incorporates some transmission, both horizontal and vertical, between host species. The rate of horizontal transmission in this study is numerically so low as not to noticeably affect population dynamics, but the vertical transmission rates result in high absolute numbers of hosts entering and leaving the partial refuges created by the secondary endosymbiont infection. Little is known about the possible mechanisms of horizontal conspecific transfer of endosymbionts. However, given that variation of the horizontal transmission rate in this study has only a small, or indeed negligible, effect on the overall population dynamics, gaining a better understanding of other parameters for which there is scarce experimental evidence, such as the induced cost of harbouring an endosymbiont infection, is more critical to this study.

Returning to the phenomenon of superparasitism, endosymbiont-mediated resistance to parasitism is analogous to partial refuge from predation. Hosts maintaining protection after first parasitoid attack can prevent over-exploitation of hosts. Again, there is a balance between parasitoids holding hosts below carrying-capacity and overall population extinctions. In a study focused on parasitoid behaviour using uncharacterised hosts, Bai (1991) asserts that *A. ervi* chose to preferentially oviposit in unparasitised hosts when given a choice between parasitised and unparasitised hosts. When host choice was removed and parasitised hosts offered, pea aphids that had been parasitised more than $24\,\mathrm{h}$ earlier were rejected but recently parasitised hosts accepted. The explanation offered for this is that time-limited *A. ervi* wasps are less selective about host status than other species, such as egg-limited *A. asychis*.

It was intended to carry out a global stability analysis as part of this study, but no suitable computational package could be sourced to deal with the discrete equations used, and analysis using mathematical methods was beyond the scope of this study due to the complexity of the model equations.

Modelling the same interactions using a different mathematical technique would allow for an assessment of the robustness of the model. If similar conclusions were

found, then this model of the biological study system could be considered more robust. Aside from the discrete approach, differential equations are often used to describe such systems and individual based modelling approaches are becoming more commonplace. With regard to parameterisation of this, or any future model, Morgan et al. (2001) assert that performance data from different areas must be used judiciously in population forecasts due to the variation in life history parameter values calculated between studies. They report findings from the literature that suggest parameter variation may be due to different rearing conditions, but caution that other studies assert that variation may be due to adaption to local climates.

5.4 Summary and conclusions

The anomalies identified in the Kwiatkowski and Vorburger (2012) model coding did not occur in the revised model presented in this study. Sweeps through $c_c \times p_s$ parameter space with different induced costs of harbouring a secondary endosymbiont infection showed that uninfected and infected host populations could coexist in a stable manner for (approximately) low to midrange constitutive costs and midrange to high strengths of protection. Stability boundaries were identified. As the induced costs increases, the boundaries between oscillating and stable populations, and between coexistence and persistence of uninfected hosts only shifted towards lower cost and higher benefits, and the amount of coexistence decreased.

Time delays were shown to be responsible for some of the oscillatory behaviour in the model output. Changing horizontal transmission rates had no noticeable effect on stability boundaries; changing vertical transmission rates shifted stability boundaries to lower strengths of protection for the same costs.

Decreasing the strength of protection provided by the endosymbiont to pea aphids on second and subsequent attack decreased the fractional amount of coexistence between uninfected and infected host populations. When parasitoids were permitted some choice over which hosts to attack, the amount of coexistence between host classes again decreased as induced costs increased, but to a lesser extent. The region of stable coexistence extended to all strengths of protection when constitutive costs were low. The maximum degree of suppression of stable host populations by parasitoids was approximately $q = 0.2$, i.e. reduced to 20% of carrying capacity. Stable coexistence uninfected and infected host populations ex-

isted, held below carrying capacity by predation by natural enemies, at plausible parameter values for the costs and benefits in the *A. pisum*-*A. ervi* system.

Computational methods developed during the course of the exploration of the Kwiatkowski and Vorburger (2012) model, provided a way of reproducing the results of Godfray and Hassell (1991). An analogy was drawn between the strength of protection afforded by endosymbiont infected and the safety of a partial refuge from predation. It was shown the the evolution of encapsulation with resource limitation model presented by Godfray and Hassell (1991) could be used to produce similar results, albeit with a simpler model, to the sweeps through parameter space reported by Kwiatkowski and Vorburger (2012).

6 Summary of findings and future perspectives

6.1 Summary

All 16 pea aphid clonal lines held in culture at JHI harboured the primary endosymbiont *Buchnera*. From PCR screening, 14 lines were found to harbour 1 or more of the known pea aphid secondary endosymbionts. There were 2 secondary endosymbiont free lines, 1 line infected with *H. defensa* only, 3 lines with the double infection *H. defensa* with PAXS and 1 line infected with single infection PAXS. Pea aphids infected with both *H. defensa* and PAXS are highly resistant to parasitism by parasitoid wasps (Guay et al., 2009) but the role of PAXS is not known and the mechanism by which resistance is increased has not been explained. Although pea aphids harbouring a single infection of PAXS have been previously identified in the published literature (Ferrari, 2011; Henry et al., 2013), this study is the first to begin to test the susceptibility of such aphids to parasitoid wasps. APSE was found in 6 pea aphid clonal lines that also were found to harbour an *H. defensa* infection and the PCR primers targeting the highly conserved P35 APSE gene gave consistent results across all APSE infected pea aphid lines. The pea aphid lines held at JHI were genotyped using microsatellite markers for the first time and all pea aphid clonal lines held at JHI were found to be genotypically distinct.

Analysis of previously gathered data showed that the difference in survival between aphid endosymbiont infection status (when grouped as *H. defensa*, *H. defensa* with PAXS, unknown or none, and *S. symbiotica*) was significant. Aphids infected with the double infection *H. defensa* with PAXS lived for a significantly shorter time than other aphid lines. A new experimental method of rearing pea aphids in petri dishes rather than culture cups was developed and used to measure the average time for uninfected pea aphid from line LL01 to reach adulthood (7.93 ± 0.46 d) and to reach reproduction (9.73 ± 0.59 d) under growth room conditions where

pea aphids are reared after parasitism assays.

Statistical analysis of preliminary assays to assess the susceptibility to parasitism by *A. ervi* parasitoid wasps of pea aphids harbouring single infections of PAXS and dual infections of PAXS with *H. defensa* showed that, despite visibly lower mummy counts in PAXS infected pea aphid lines, there was no evidence to reject the null hypothesis was that there is no difference in susceptibility to parasitism between lines harbouring *H. defensa* and the uninfected lines used in the assay. Additionally, there was insufficient evidence to reject the null hypothesis that there was no difference in susceptibility to parasitism between lines harbouring PAXS and the uninfected lines because there was a significant interaction between clonal line and replicate. These initial results are of interest as nothing is known in the published literature about the susceptibility of single PAXS infected aphids to parasitism by *A. ervi* wasps and suggest that this line of enquiry merits further investigation.

A mathematical model of the *H. defensa-A. pisum-A. ervi* system was developed from the work of Kwiatkowski and Vorburger (2012). Problems with the model coding provided by Kwiatkowski and Vorburger (2012) were identified and overcome in the revised model used in this study. Further, computational methods for finding stability boundaries, and boundaries between extinction and coexistence, were developed and applied. Contrary to the findings of Kwiatkowski and Vorburger (2012), sweeps through $c_c \times p_s$ parameter space with increased induced costs of harbouring a secondary endosymbiont infection showed that uninfected and infected host populations could actually coexist in a stable manner for (approximately) low to midrange constitutive costs and midrange to high strengths of protection. As the induced costs increases, the boundaries between oscillating and stable populations, and between coexistence and persistence of uninfected hosts only, moved to lower cost and higher benefits and the amount of coexistence decreased.

The model incorporates some biological realism and part of this was the inclusion of the time for a wasp to kill an aphid host and the time for the next generation of wasp to emerge from a dead aphid host. These time delays were shown to be responsible for some of the oscillatory behaviour in the model output.

Horizontal conspecific endosymbiont transmission at a low rate by an unknown mechanism was included in the model. Vertical maternal transmission from mother to nymph at high fidelity was also part of the model. Whilst varying horizontal

transmission rates had no visible effect on stability boundaries, varying the vertical transmission rates shifted stability boundaries to lower strengths of protection for the same costs.

As a broad indication of the potential effects of superparasitism, decreasing the strength of protection provided by the endosymbiont to pea aphids on second and subsequent attack was investigated and found to result in decreased fractional coexistence between uninfected and infected host populations. It has been suggested that parasitoid behaviour can evolve to overcome the benefits afforded to the aphid hosts. When parasitoids were permitted some choice over which hosts to attack, the amount of coexistence between host classes again decreases as induced costs increased but to a lesser extent. The previously identified area of stable coexistence widened to include all strengths of protection when constitutive costs were low. Host populations were suppressed by parasitoids to, at most, 20% of carrying capacity. Importantly, stable coexistence uninfected and infected host populations existed with populations held below carrying capacity by predation by natural enemies, at biologically reasonable parameter values for the costs and benefits in the *H. defensa-A. pisum-A. ervi* system.

An analogy between the strength of protection afforded by endosymbiont infection and the safety of a partial refuge from predation was made by Godfray and Hassell (1991) and they discuss the balance between coexistence and overexploitation, and, hence, extinction of host populations. It was shown that the computational methods developed in this study could be applied to the more basic evolution of encapsulation with resource limitation model (when a "mutant" strain of host akin to an endosymbiont infected host is introduced) presented by Godfray and Hassell (1991).This produces broadly similar results to those reported by Kwiatkowski and Vorburger (2012) when sweeps were carried out through cost-benefit parameter space.

6.2 Future perspectives

6.2.1 Molecular work

Pea aphids with a double-infection of *H. defensa* with PAXS have been reported as highly protective in the published literature, and also have been seen to have provided total protection against parasitism and superparasitism by *A. ervi* wasps

in exposure assays carried out on clonal lines N116 and N198 at The James Hutton Institute (*pers. obs.* and Cornwell (2011)). Wasp eggs are being deposited in the N116 and N198 lines when pea aphids are attacked (*pers. obs.* and Donald et al. (2016)). As little is known about the costs and benefits to pea aphid lines of harbouring a single infection of PAXS and, although there was a significant interaction between endosymbiont infection and replicate in the preliminary exposure assays carried out as part of this study, there is sufficient evidence to merit further investigation of such singly infected lines, as a PAXS infection may confer protection against parasitism and super parasitism by *A. ervi* wasps. Sequencing all the clonal lines that yielded positive results for PAXS during the diagnostic PCR carried out during this study (lines JF201, N116, N198, and KD13/02) would confirm if all lines harbour the same PAXS endosymbiont. As the protection afforded to pea aphid lines harbouring *H. defensa* without PAXS to parasitism by *A. ervi* has been reported to vary, it would be interesting to see if sequencing results show that the double-infected lines with PAXS have the same PAXS endosymbiont as the KD13/02 single-infected line. If so, a future hypothesis to be tested is that the extra protection is due to the infection with the PAXS endosymbiont. It would be also interesting to sequence the transient *Rickettsiella* product of unknown origin that was detected in JF200 pea aphid clonal line sample to see how it compares to the known secondary endosymbiont.

Degnan and Moran (2008) assert that a Southern blot or amplification of intergenic spacers are necessary to confirm the presence of the APSE bacteriophage, and it would be prudent to screen the pea aphid clonal lines using at least one of these methods. If intergenic spacing is chosen as a method, new forward and reverse primers for the P3 gene should be purchased and tested, as no product was obtained during PCR amplification using previously purchased primers for this gene. As a number of variants of the APSE bacteriophage have been identified in the published literature, identification of the particular strain infecting the *H. defensa* pea aphid clonal lines at The James Hutton Institute could help inform future work looking into the strength of protection of *H. defensa* lines, as there is 1 pea aphid clonal line at JHI that is only infected with *H. defensa*, and the preliminary exposure assay gave no evidence to reject the null hypothesis that there was no difference in susceptibility to parasitism between lines harbouring *H. defensa* and the uninfected line.

There is increasing interest in the role of resistance to parasitism that is geno-

typic in origin. Although all pea aphid lines at JHI have been identified as genotypically distinct, the primer for the AlB08M allele gave unsatisfactory results compared to other primers and there was no variation between clonal lines at the ApH04 allele. Although diagnostic PCR using primers for the microsatellite alleles identified by Caillaud et al. (2004) yielded correctly sized DNA products in 5 out of 13 primers tested, there are two further primers listed that have not been tested. The next stage in identifying more suitable primers than AlB08M and ApH04M for genotyping the pea aphid clonal lines would be to test the given primers for the AlA12M and AlB07M microsatellite alleles modifying the thermocyling conditions to use an annealing temperature of $60\,^{\circ}$C.

There was no rigorous attempt to quantify the resistance of endosymbiont free pea aphid line JF200 during this study, although it was noticed repeatedly that this line appeared potentially highly resistant to parasitism by *A. ervi* wasps. During dissections to look for the presence of wasp eggs in single-attacked and double-attacked 3 to 5 days old JF200 pea aphid nymphs, it was noted that fluid from the JF200 line was much clearer that that of the LL01, N116 and N198 lines. The fluid of the N116 and N198 lines appeared to contain a high amount of visible fat (H. Clarke, *pers. comm*, 2014 and *pers. obs.*). Given the observations of Sabri et al. (2011), an experiment to test if aphid fat content is related to survival and development of wasp eggs into larvae could be devised. The results of this may yield some insight into potential mechanism for genotypic rather than endosymbiont-mediated resistance to parasitism.

Another avenue for future investigation is to cure various pea aphid clonal lines of their endosymbiont infection to see if a baseline measure of innate resistance due to genotype alone can be made. This would be of particular interest for the single-infected *H. defensa* line N127, the double-infected *H. defensa* with PAXS lines N116, N198 and JF201, and the single-infected PAXS line KD13/02. Curing could be done by microinjection of pea aphids with antibiotics (Clarke, 2013), followed by diagnostic PCR of subsequent generations to check that the clonal lines were free of endosymbiont infection.

6.2.2 Insect work

6.2.2.1 Measuring the time to reach adulthood, reproductive rate and lifespan of two uninfected pea aphid genotypes.

The effective aphid fecundity is given by the number of offspring produced in a time equal to the pre-reproductive time. Previous experiment (Cornwell, 2011) showed that the reproductive rate for pea aphid line LL01 was nearly constant over the time equal to the pre-reproductive rate, slowing from the onset of reproduction to a lesser extent than for other clonal lines. An assumption made for the mathematical model was that aphid reproduction remained constant over aphid lifetime. The experiments to measure the time to reach adulthood and reproduction of uninfected pea aphid line LL01 could be repeated and extended to include the potentially genotypical resistance line JF200. Aphid lines LL01 and JF200 are genotypically distinct and stock cultures are free of any known endosymbiont infection. Rather than culling the aphids after they reach adulthood, the time to reproduction and the reproductive output of these aphids over their complete lifespan can then be measured to give improved parameter values for host birth rate and host death rate in the mathematical model.

The hypothesis to be tested would be that the time to reach adulthood, time to reach reproduction, reproductive rate and lifespan of uninfected pea aphid lines LL01 and JF200 under the same environmental conditions will be the same. The null hypothesis would be there is no difference in measured fitness parameters between these 2 pea aphid lines. It would be impractical to carry out the experiment rearing aphids in dishes due to the number of dishes required and the need to transfer aphids to fresh dishes as the experiment progressed and bean leaves deteriorated, so the experiment could be carried out on *V. faba* plants in the growth room instead. One late instar or adult apterous aphid from each line could be transferred to the underside of a recently expanded leaf of a *V.faba* plant in a labelled pot and secured using a 25 mm clip cage. This aphid is defined as generation zero. The aphid will need to be checked daily and removed after 24 h of nymph production. Excess nymphs should be removed to leave a maximum of 3 first generation nymphs in the cage. After 3 d to 4 d, nymphs should be culled to 1 per clip cage and checked daily. The day in which the nymph was seen to have become an adult (identified by the shape of the cauda) should be recorded. This is the development time to adulthood. The day in which offspring is first

observed should be recorded. This is the time to reproduction and equal to the pre-reproductive period of the aphid. Nymphs produced by these adult aphids are second generation nymphs. Offspring should be counted and removed on at least every second day until the first generation adult dies. The plants with clip cages should be kept in a growth room at $20\,^{\circ}$C and $16\,$L: $8\,$D. Three replicates of the experiment could be carried out on 1 plant per line at the same time. The whole assay should be repeated until there is no significant statistical interaction with replicate using bean plants from successive sowings.

The mean pea aphid time to adulthood, time to reproductive, reproductive rate, birth rate per day, lifespan and death rate with associated 95% confidence intervals for the 2 clonal aphid lines should be calculated, and analysis of variance (ANOVA) used to investigate the differences in fitness parameters. Replicate should be included in the analysis as a factor to account for any variation within the controlled environment rooms and plant condition, and results determined significant with a probability of $p < 0.05$.

6.2.2.2 Preliminary experiments to investigate the functional response of parasitoid wasps attacking pea aphids

In the published literature, the terms attacked and parasitised are sometimes used interchangeably to describe encounters between a parasitoid wasp and its aphid host. A common assumption is that all attacks result in oviposition and lead to successful parasitism of the host. Because the handling time of *A. ervi* is short compared to other types of parasitoid wasps, it is not possible to discriminate visually between parasitoid attacks resulting in deposition of an egg and those attacks that do not. Further, the number of mummies resulting from exposure of aphid hosts (under very variable experimental conditions) to a parasitoid wasp is used to form conclusions about the resistance of hosts to parasitism without knowledge of how many attacks took place, how many attacks would have been expected to take place, and whether any wasp eggs were indeed oviposited. It is also sometimes assumed that parasitoids attack and oviposit equally when provided with hosts regardless of host age, fitness and secondary endosymbiont infection status. A variety of experimental set-ups have been used to assess susceptibility of aphid host to parasitism from test-tubes to petri-dish arenas, to whole plants and to field/greenhouse plots containing many plants. A variety of time-scales over

which hosts have been subjected to parasitoid exposure have also been used ranging from minutes to days and a variety of host densities have been used. Some experiments restrict a parasitoid to single attack only, other experiments allow for the possibility of multiple attacks upon the same host and hence superparasitism. Knowledge of the expected rate of host attack when exposed to a parasitoid under particular experimental conditions is necessary to assess more rigorously whether differences in parasitism success rates in future experiments using the same set-up are indeed due to variation of host resistance, rather than due to the functional response and behaviour of the parasitoid under such conditions. The clonal lines of key interest here are LL01 (endosymbiont free and genotypic low resistance to parasitism), JF200 (endosymbiont free and with possible high genotypic resistance to parasitism), KD13/02 (single-infected with potentially highly resistant PAXS endosymbiont), and N198 (double-infected with *H. defensa* and PAXS endosymbiont and known to be highly resistant to parasitism).

Preliminary assays could be carried out to improve the protocol for future arena parasitism experiments and to investigate the attack rate of and deposition of wasp eggs into pea aphids by parasitoid wasps. After attacks, most nymphs will be left to develop into mummies. The fitness of wasps emerging from these mummies could be assessed to test the assumption in the mathematical model that wasps take an equal time to emerge for uninfected and infected hosts, and that wasps emerging from infected hosts remain capable of attacking aphids at the same rate as wasps emerging from uninfected hosts. Pea aphid nymphs could be dissected to test the mathematical model assumption that each attack leads to deposition of an wasp egg regardless of infection status.

To generate pea aphid nymphs for each assay, apterous adult and late instar LL01 pea aphids should be transferred onto a *V. faba* cutting in a culture cup. Aphids should be allowed to reproduce for 36 h and cultures placed in the insect rearing room and maintained at $18\,^{\circ}$C, 16 L: 8 D. After 36 h, adult aphids should be removed and nymphs left in the culture for a further 3 d, by which time they will be 2nd and 3rd instar nymphs. Experimental arenas should be prepared by fixing bean leaves onto agarose gel in petri dishes. For each replicate, 5, 10, 20 and 40 nymphs will be transferred to an arena and left to settle for 1 h. A single female *A. ervi* parasitoid wasp (assumed previously mated) aged between 2 d to 5 d will be introduced to the arena and left to forage for 30 min. Any wasp failing to attack within 5 min of start of assay should then be replaced with a fresh wasp.

The wasps should be reared on *A. pisum* clonal line LL01 and separated into day cohorts upon emergence. The number of wasp attacks on pea aphids in the arena during the assays should be recorded. The nymphs should be retained in the dish after the assay, but the wasp removed from the arena. Petri dishes should be left in a growth room at $20\,^{\circ}$C and 16 L: 8 D. A proportion of nymphs will be dissected to check that oviposition has taken place. After allowing nymphs to settle for 48 h, the remaining aphids from each petri dish should be transferred into separate culture cups or dishes containing a fresh *V. faba* cutting. The number of adult or fourth instar aphids present in each cup should be counted after a time interval (from the end of nymph generation) equal to the time to reach adulthood for an LL01 aphid and these aphids should be removed from the cup. The number of mummies should be counted 12 d to 14 d after initial introduction of the parasitoid wasp. The mummies should be removed from the culture cup and transferred to a box containing cotton wool soaked in dilute honey. The box should be checked daily and any wasps emerging removed and stored in ethanol in a labelled Eppendorf tube, and the time taken to emergence, host density and host line noted. Wasp fitness should be measured. This procedure should be replicated using *A. ervi* parasitoid wasps from different generations until there is no significant statistical interaction between variables and replicate.

The mean number of attacks at each host density can be plotted against host density on a scatter plot. Linear regression can be used to establish the type of functional response (Hollings type I, II or III) and to estimate the parameters relating to searching and handling time associated with that functional response. These results will inform choice of host density and assay duration in the future experimental assays. Further replicates at intermediate and higher host densities can be carried out if necessary if there are parts of the graph where the shape of the functional response is unclear. The mean wasp emergence time and parasitism success rate with associated 95% confidence intervals for the LL01 aphid line should be calculated, and analysis of variance (ANOVA) used to investigate the differences between number of attacks recorded and number of mummies resulting. Replicate number should be included in the analysis as a factor to account for any variation within the controlled environment rooms and/or wasp generation. Results will be determined significant with a probability of $p < 0.05$.

The use of the Observer system could be trialled and, in addition to attacks, used to record wasp behaviours (searching, oviposition attempts (successful, failed),

grooming) and aphid behaviours (kicking, running away, honeydew excretion). The output from the Observer software could yield useful information about the timing and frequency of attacks, wasp search time and pauses between attacks. It would be prudent to check that flicker from fluorescent lighting in the growth room was not affecting insect behaviour. To establish suitable times for aphid dissections to confirm oviposition, assays could be carried out with 40 nymphs aged 3 d to 5 d from which cohorts of 10 nymphs are dissected from 6 h to 8 h, 1 d, 2 d and 3 d after attack to look for evidence of wasp eggs or larva. The number of eggs/larvae per host should be recorded. Each assay should be carried out twice at each density for each line LL01, JF200, KD13/02 and N198, with aphids from 1 replicate dissected as described and aphids from the other replicate left to develop into mummies. Lines and outcomes (dissection/mummies) should be randomly allocated within time block.

6.2.3 Modelling

Snyder and Ives (2003) use a stage-structured matrix model to study the interaction between pea aphids and *A. ervi* wasps. They explicitly model the developments of both aphid hosts and parasitoid wasps through the various instar development stages until adulthood, and consider the relevant factors affecting population growth, survival and parasitism at each life history stage. Unlike Kwiatkowski and Vorburger (2012), the emphasis of Snyder and Ives (2003) is on the relationship between generalist and specialist predators upon aphids within a biocontrol context, however there is some overlap in the class structure used, although the mathematical approach taken is different. It may be possible to develop the approach taken by Snyder and Ives (2003) to include the presence of protective facultative endosymbiont infection in the aphid populations. As with all modelling, a judgement call must be made on the level of detail included within the model. With the Kwiatkowski and Vorburger (2012) model as it stands, there has been some attempt to include factors such as parasitised aphids continuing to exploit resources until death (albeit at a very basic level which assumes a parasitised aphid 1 day from death behaves the same as any other aphid) and hence contribute to density-dependent host survival, however, other details such as the nature of the parasitoid functional response has been lost as a consequence of the way in which the model is expressed. It could, perhaps, be argued that the functional response

is of more interest overall with regard to biocontrol applications. The Kwiatkowski and Vorburger (2012) model also lacks the finer detail of preferential parasitism of aphid instar phases that the Snyder and Ives (2003) paper contains. Given the complexity of the Kwiatkowski and Vorburger (2012) model, it was not unreasonable of the authors not to include such detail as the mathematics and coding of their paper is already complex.

In future modelling work, the assumptions made about aphid reproduction may be reconsidered. Both Kwiatkowski and Vorburger (2012) and this study ignore the potential reproductive output of aphid hosts parasitised as adults or when close to adulthood. It has been noted during experimental work that such aphids are capable of continuing reproduction for a while. Further, aphid reproductive output has been assumed to be constant. Typically, aphid reproductive output rises from the onset of reproduction to a peak at $2\,\mathrm{d}$ to $3\,\mathrm{d}$ into adulthood. The subsequent decrease in reproductive rate can be affected by environmental conditions. Environmental factors may also affect the expression of the costs and benefits of harbouring an endosymbiont infection. There has been some work carried out on temperature effects (Guay et al., 2009) but there is not much reported work in this area in the published literature. The costs of harbouring an infection, both constitutive and induced, are currently expressed through constraints on the aphid host birth rates. Data for infected pea aphid lines held at JHI show a difference in survivorship between lines with single and double infections of *H. defensa* and *H. defensa* with PAXS. Results of future aphid fitness experiments may be used to refine and inform the model, perhaps modifying the host death rate(s) appropriately. Similarly, it may be that any difference in wasp fitness between adult wasps emerging from differently infected aphid hosts may be incorporated into the model. Some measure of the importance of each of these effects on the model output would be helpful.

Corley and Capurro (2000) present a modified Nicholson Bailey model taking into account prolonged diapause, introducing a delay in the emergence of adult parasitoids. The time interval used in the model is, however, yearly generations rather than daily changes in population size. This model mirrors the delay in parasitoid emergence corresponding to the time to kill and emerge from a parasitised aphid in the Kwiatkowski and Vorburger (2012) model, but the time scales vary. The persistence of long term oscillatory dynamics upon introduction of the delay is noted. Corley and Capurro (2000) discuss whether it is useful to emphasise

local stability in a biocontrol context. Given the huge variation in modelling approaches taken, and the wide interpretation of terms such parasitism rate, direct comparison of results from different studies is difficult. In addition, given the lack of knowledge about the effects of protective endosymbiont infection on parasitism rates, few papers prior to the discovery of the role of *H. defensa* make reference to pea aphid lines used in studies, and the infection status of most lines is unknown. Hence the conclusions reached are reported as general findings, but may be particular to the specific lines, susceptible or otherwise, used in the study. More recently, pea aphid lines used in experiments have started to be genotyped. Again, general results reported in the literature prior to the advent of such genotyping must be regarded with caution when comparisons are made between studies. Further, this study does not extend to the introduction of selective pressure upon either host or parasitoid.

Given the large number of variables involved, it is challenging to formulate a meaningful hypothesis that can be tested both mathematically and experimentally. Bringing together modelling and experimental work is important but this often generates more questions. For example, is a measured population increase over time definitely due to experimental manipulation of another variable, or is it the biological study system finding its equilibrium? Are the fluctuations found in field data over a short time interval simply the initial transient dynamics? When does the system ever get a chance to settle in the field? Can information be gleaned from modelling simulations for a number of years on a computer be compared to data punctuated by seasons in the field? Can simulations on a grid/experiments in a petri dish be used to inform predictions in the field? Other factors come into play in the field, such as seasonality, host plant specificity and parasitoid emigration/immigration. It is critical to identify an area of focus and make suitable predictions. Risking a subjective judgement, it feels as if the "why" lags behind the "how" in this area of modelling. Too often, a scenario is modelled, and then the biological question tacked on the end, rather than a clear, testable biological hypothesis being identified at the start. Hence, there is unfortunate conflict between the mathematics and the biology. However, this is not unique to this area of study. The purpose of the model needs to be explicit; is the model descriptive or predictive? Does adding more complexity to the model necessarily lead to a better understanding of the system or a more useful forecast? Importantly, how can the model output be tested against experiment?

Much of the modelling work in this study has followed the approach taken by Kwiatkowski and Vorburger (2012). There are short-comings in trying to model some of the intricacies of host-parasitoid interactions using a model that considers only populations rather than individuals. The advantage of trying to refine and build upon the body of work generated by such a model is that results and conclusions can be compared to findings in the published literature. This enables previous conclusions to be possibly challenged, and avenues for future research identified. However, the lack of individual traceability within the model when some of the parameters arise from individual host-parasitoid interactions is a disadvantage. Given more time, exploration of the same basic model interactions using other approaches such a matrix-models or individual based models would be beneficial. However, a clear focus for the study would be needed. One approach taken in the published literature is the use of integrodifference models to study population host-parasitoid population dynamics. There are drawbacks to applying such an approach to the system modelled here when the spatial component is introduced into the model if comparison of conclusions are required. The protection conferred by endosymbiont infection has been discussed earlier as analogous to a partial refuge from predation. When considering spatio-temporal dynamics, the concept of partial refuges again arises, but this time as physical refuges from predation. Host-parasitoid dynamics have been modelled using a variety of methods including integrodifference equations (Sherratt, 2001; Sherratt et al., 1997, 1995). A specific difficulty in trying to code the model in terms of integrodifference equations is how to keep track, in space, of dying aphid hosts and emerging parasitoid wasps.

Recognising the difficulties that seasonal variation brings to modelling host-parasitoid interactions, Briggs et al. (2004) review the development of hybrid models potentially applicable to the *A. pisum*-*A. ervi* biological system. The two elements in this type of model are a between-season component and a within-season component. The between-season component carries forward the population densities from one season to another and incorporates a seasonal pulse of reproduction. The within-season part of the model includes such processes as parasitism, death and dispersal. Such a model could be framed to reflect the nuances of this biological study system, in the field in the UK, where multiple generations under summer conditions are interspersed with overwintering periods for the pea aphid hosts and diapause for parasitoids. The authors note that such an approach is unwieldy and

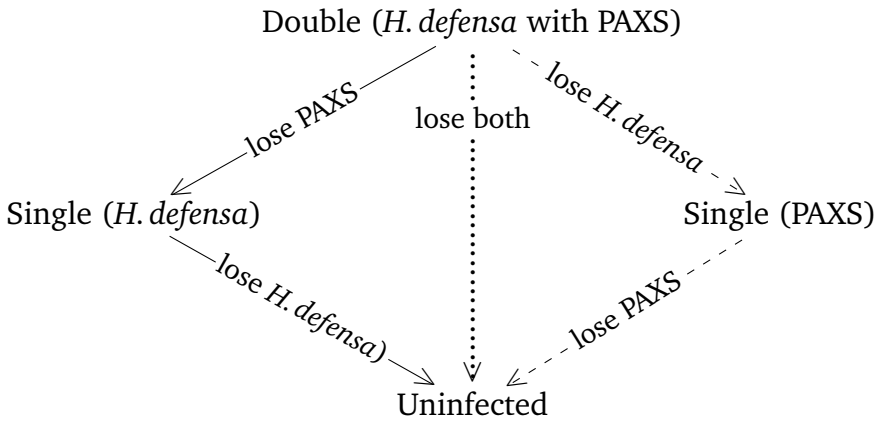
reliant on numerical simulation. However, it is a potential avenue for the inclusion of further biological realism into the modelling process and it would be feasible to include the endosymbiont-host interaction within such model.

Begon and Mortimer (1986) debate the validity of an equilibrium view of community organisation. Presenting both equilibrium theory that stresses the importance of the dynamical system studied at and near its equilibrium point and nonequilibirum theories that consider transient system dynamics, the authors acknowledge that, in the field rather than in the laboratory or simulation, the likelihood of a system reaching its steady state is far from certain due to a myriad of spatio- and temporal fluctuations. They report, albeit somewhat hopefully, that researchers directing their effort and attention towards the equilibrium are mindful of the oscillatory behaviour of the system to a varying degree around this point, and that the boundary between equilibrium and non equilibrium theories is at best blurred. However, focus on temporal variation can be very informative.

Briggs et al. (2004) also discuss the merits of a focus on stability in two-species host-parasitoid models. According to research reported by the authors, the lack of success of a biological control programme has not once been attributed to the absence of stability in the biological system, but instead failure is due either to the introduced natural enemy (parasitoid) population not establishing itself or not adequately lowering the pest (host) population. Whilst recognising that considerable attempts have been made to improve the biological realism of theoretical models, the authors stress the economic importance of biological control programmes that hold pest populations at a low enough level to prevent outbreaks, and identify the dearth of experimental test and verification of theoretical modelling work as a serious shortcoming in this area of research.

This study does find that uninfected and infected host populations may coexist in stable equilibrium as lowered population densities within the range of biologically likely constitutive costs and beneficial endosymbiont conferred protection. In addition to the stabilising factors discussed in the published literature, it seems from the evidence presented in this study, that multi-trophic interactions between microbial endosymbionts, aphid hosts and parasitoid wasps are worthy of further investigation as both stabilising factors in the host-parasitoid system and as viable drivers of reduced prey density in biological control scenarios. In deciding how to go about tackling the mismatch between experiment and theory, the final product produced by the modelling process should more closely inspected within this

Figure 6.1: Vertical transmission between model classes: adding a PAXS infection.



overarching context.

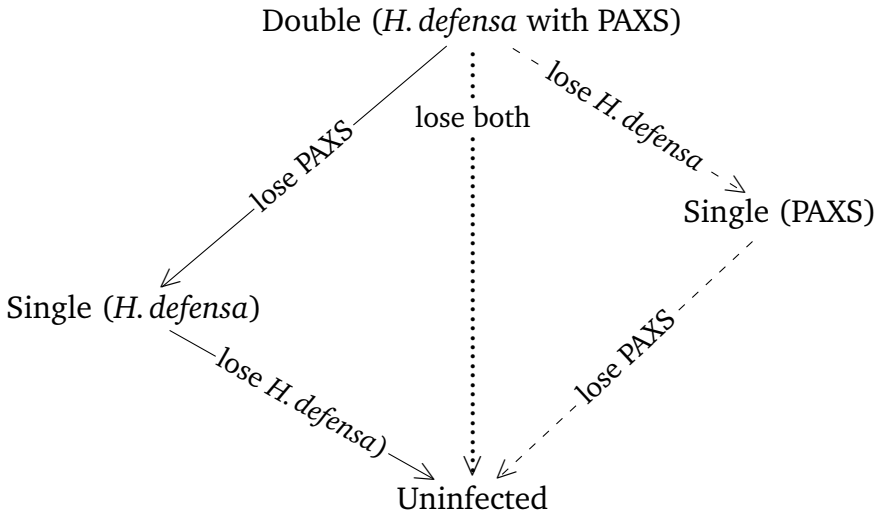
6.2.3.1 Modelling single and double endosymbiont infections within a single genotype

Rather than stipulate a specific mathematical approach to further modelling, this study uses information gained from study of the biological system in relation to the dual *H. defensa* with PAXS infection, and from the intriguing hints at genotypic variation in resistance to parasitism, and proposes future changes to the host categories used in the modelling work.

A dual-infection of *H. defensa* with PAXS has been shown to give pea aphids full protection against parasitism by *A. ervi* wasps. Introducing PAXS infected pea aphids to the model adds a single-infected PAXS class and a double-infected *H. defensa*-PAXS class (figure 6.1).

The resistance to parasitism of pea aphids infected with PAXS is yet to be established, although preliminary experiments suggest that this infection may be protective. If the protection provided by PAXS is higher than that provided by *H. defensa* or is retained upon superparasitism (unlike *H. defensa*), the parity between single infected classes in the model changes (figure 6.2). This can be reflected in the model by suitable choice of parameter values for the protection provided by each endosymbiont. The change in parity should be informed, therefore, by experimental findings. The probability of a newly born aphid losing an endosymbiont

Figure 6.2: Vertical transmission between model classes: is PAXS more protective than *H. defensa*?



in the next generation is assumed to be the same as before.

If, in the model, resistance to parasitism is contingent on infection with *H. defensa*, then pea aphids infected with PAXS only are considered to have the same status as uninfected aphids (figure 6.3). Again, the probability of a newly born aphid losing an endosymbiont in the next generation is assumed to be the same as before. However, it should be noted that there are two routes into the lowest (unprotected) pea aphid class of the model which contains uninfected and PAXS-only infected pea aphids.

Sources of *H. defensa* and PAXS horizontal transmission between model classes are explored in figure 6.4. The diagram shows the separate routes of conspecific infection of the PAXS endosymbiont and of the *H. defensa* endosymbiont from each infected pea aphid host class to another pea aphid host not currently harbouring that particular microbial association.

Figure 6.3: Vertical transmission between model classes: protection to due *H. defensa* only.

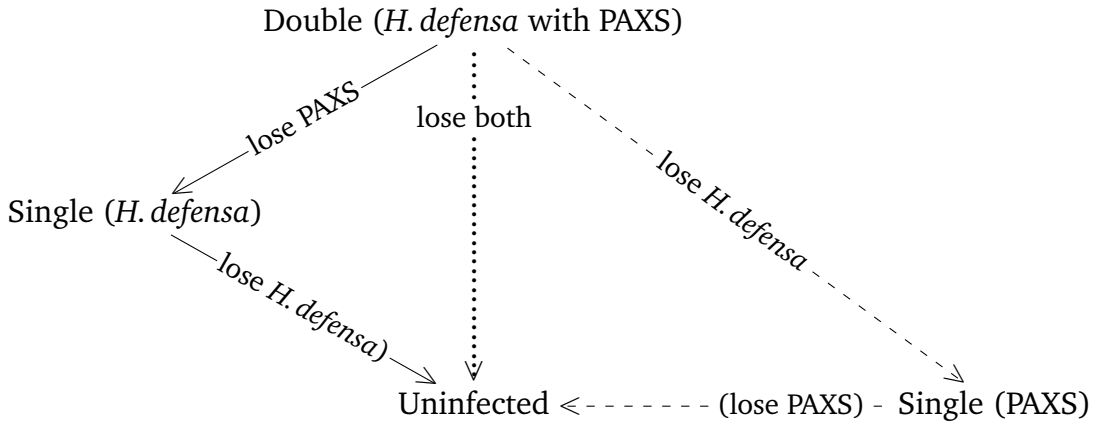
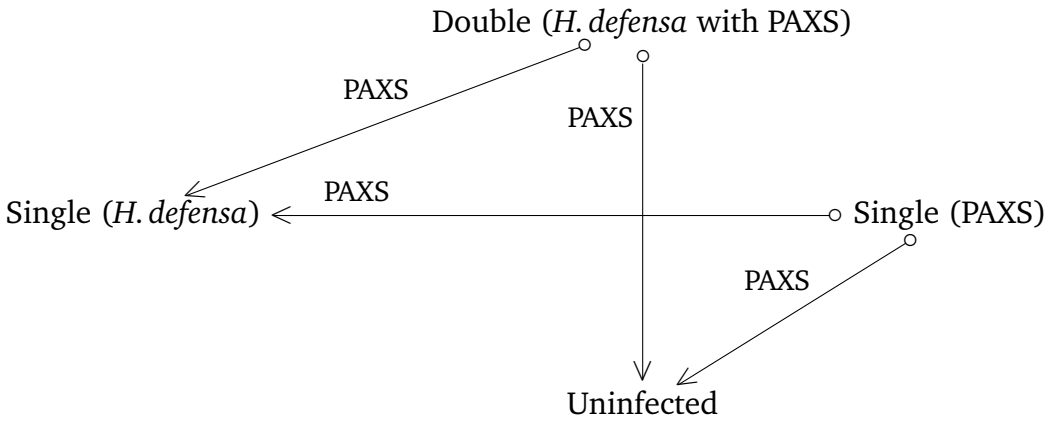


Figure 6.4: Sources of PAXS (top) and *H. defensa* (bottom) horizontal transmission between model classes.



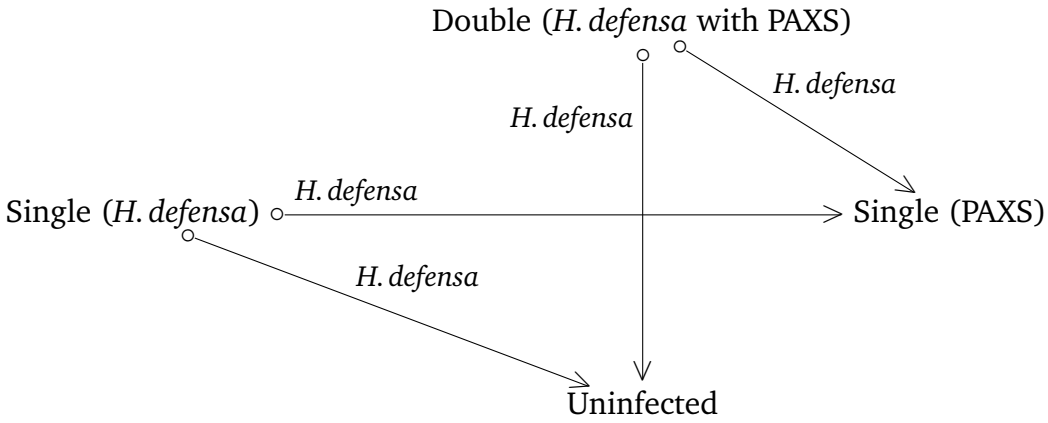
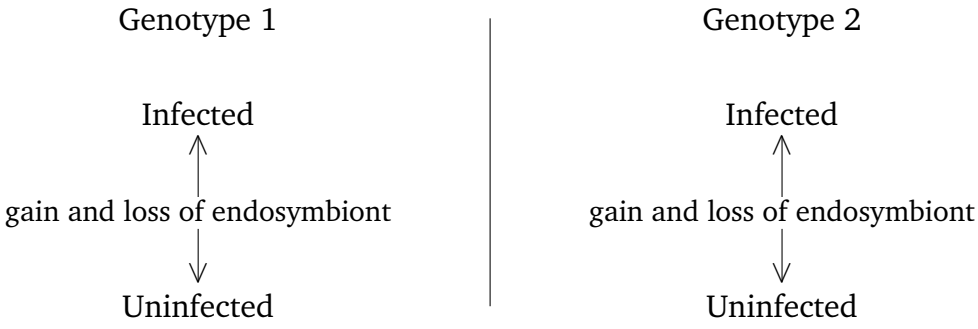


Figure 6.5: Model classes: genotypic variation in innate resistance only.

Figure 6.6: Model classes: genotypic variation with endosymbiont infection.



6.2.3.2 Modelling the introduction of an additional pea aphid genotype

Before further complicating the interactions between classes in the model, it may be worth looking at innate resistance to parasitism only to see what effect this has on the model output. The simplest case of genotypic variation between pea aphids can be modelled by considering two uninfected pea aphid classes of different genotype, as shown in figure 6.5. There is experimental evidence of an endosymbiont-free pea aphid line with a high resistance to parasitism, JF200, compared to the uninfected line LL01 (*pers. obs.*). Differences in resistance to parasitism are innate to each pea aphid genotype. A model with this reduced complexity may also be more accessible to analysis by analytical means in addition to numerical simulations.

Introducing an endosymbiont infection yields the model classes shown in figure 6.6. One possibility is that an infected aphid of either genotype can acquire an endosymbiont horizontally, as shown in figure 6.7. However, as there might be genotypic resistance to endosymbiont acquisition, it is possible that infected aphids of one genotype cannot transmit an endosymbiont horizontally as shown in figure 6.8 and the two populations remain isolated from each other.

Figure 6.7: Genotypic variation: sources of horizontal endosymbiont transmission.

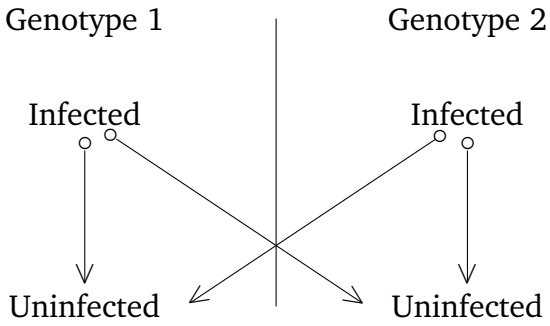
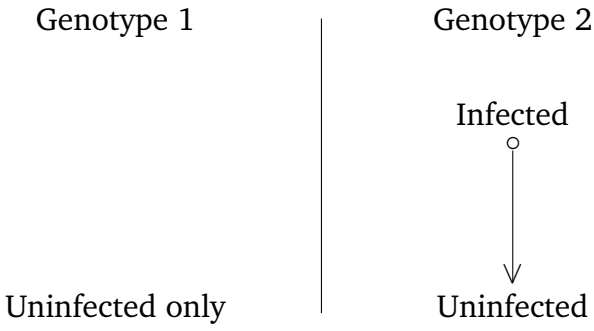


Figure 6.8: Genotypic variation: sources of horizontal endosymbiont transmission if populations remain genotypically isolated.



6.3 General conclusions

This study illustrates the intricacy of the *A. ervi-A. pisum* interaction when pea aphid endomicrobiota are factored into the host-parasitoid dynamics. It presents evidence of wide ranging associations between facultative secondary endosymbionts and their pea aphid hosts and specifically identifies a dual infection of *H. defensa* with PAXS as particularly beneficial when hosts are subject to parasitoid attack. This study further reports the discovery of a clonal line of pea aphids harbouring PAXS only. A mathematical model of the aphid-wasp host-parasitoid system published by Kwiatkowski and Vorburger (2012) is refined and developed and parameterised specifically for the *H. defensa-A. pisum-A. ervi* multi-trophic interaction, placed within a biological control context, and the findings of Kwiatkowski and Vorburger (2012) are challenged with respect to the resulting population dynamics. Further modifications to the model classes are suggested in light of the experimental work in this study.

However, the complexity of the endosymbiont-host-parasitoid interaction pales when considered within the wider ecosystem into which agricultural and horticultural biological pest control is introduced. Once placed within the food web (Messelink et al., 2012), resulting competition, intraguild predation, omnivory and hyperparasitism further convolute the interactions both within and between trophic levels. This does not necessarily diminish the importance of studying the effect of microbial symbionts on the dynamics of natural enemy resistance in aphid populations, but serves to highlight the richness of the biological system studied and the need for further work on this topic.

A The MATLAB code for the implementation of the endosymbiont-mediated protection model

The basic MATLAB population model script is:

```
%Population model

%Time
DAYS=3650;
BURNIN=365;
t=(1:1:DAYS);

% Model parameters
% Baseline values from Kwiatkowski and Vorburger (2012)
bh=2;       % Basal reproduction rate of hosts
dh=1/20;    % Host death rate
dp=1/3.5;   % Parasite death rate
tv=0.8;   % Reliability of vertical transmission
th=0.0;   % Rate of horizontal symbiont acquisition
pi=0.5;     % Innate resistance to parasitism
ps=0.6;     % Level of protection conferred by symbionts
psp=ps;     % Resistance to second and subsequent attacks 0<psp<1,
% can be fraction of ps, affects HR class, new parameter not in KV
    model
cc=0.5;     % Constitutive cost of infection
ci=0.0;     % Induced cost of infection
lk=9;       % Time to kill a parasitised aphid
le=5;       % Time to emerge from a mummy
```

```
k=15000;    % Carrying capacity of aphid host populations
% Type II functional response:
% Values from Snyder and Ives (2003) field values A. ervi on A. pisum
a=146;      % Search efficiency of parasitoid
b=0.0011;   % Handling time (b=zero for Type I functional response)

% Preallocating for speed...state equations later in MATLAB script
% State variables:
% Class 0 is uninfected, class S is single infected
% Unprotected aphid population (w/o wasp eggs/larvae, never attacked
    and
% survivors of attack):
H0=zeros(size(t));
% Protected aphid population (w/o wasp eggs/larvae, never attacked):
HS=zeros(size(t));
% Protected aphid (attack survivors, any wasp eggs/larvae fail to
    develop):
HR=zeros(size(t));
% Parasitised unprotected aphid population (still alive but going to be
% killed by parasitism):
V0=zeros(size(t));
% Parasitised protected aphid population (still alive but going to be
% killed by parasitism):
VS=zeros(size(t));
% Wasp population
P=zeros(size(t));

% Auxiliary quantities
% Unprotected aphids successfully attacked (parasitised) that day:
success_attack0=zeros(size(t));
% Protected aphids successfully attacked (parasitised) that day:
success_attackS=zeros(size(t));
% Total host population (including parasitised not yet dead aphids):
total_hosts(t)=zeros(size(t));
% Fraction of available hosts attacked:
fraction_attack(t)=zeros(size(t));
% Fraction of infected hosts for horizontal transmission of
    endosymbiont:
horizontal_acq(t)=zeros(size(t));

% Initial populations
H0(1)=800;
```

```
HS(1)=200;
HR(1)=0;
V0(1)=0;
VS(1)=0;
P(1)=20;

% Set up the delays
% Putting "lk" days worth of zeros
i=(1:1:lk);
killed0_delay=zeros(size(i));
killedS_delay=zeros(size(i));
for i=1:lk
killed0_delay(i)=0;
killedS_delay(i)=0;
end

% Putting "le" days worth of zeros
j=(1:1:lk+le);
emerged_delay=zeros(size(j));
for j=1:(lk+le)
emerged_delay(j)=0;
end

% Run model through time
for t=1:DAYS

%These auxilliary quantities need to be calculated to be used in
    equations
% for state variables
total_hosts(t)=H0(t)+HS(t)+HR(t)+V0(t)+VS(t);
fraction_attack(t)=(1-exp(-a*P(t)/(1+a*b*(H0(t)+HS(t)+HR(t)))));
success_attack0(t)=(1-pi)*fraction_attack(t)*H0(t);
success_attackS(t)=(1-pi)*(1-ps)*fraction_attack(t)*HS(t)...
     +(1-pi)*(1-psp)*fraction_attack(t)*HR(t);
horizontal_acq(t)=(1-exp(-th*((HS(t)+HR(t)+VS(t))/total_hosts(t))));

% Put delays in front of daily data for parasitised aphids:
% Unprotected parasitised aphids killed that day by parasitism:
killed0=horzcat(killed0_delay,success_attack0);
% Protected parasitised aphids killed that day by parasitism:
killedS=horzcat(killedS_delay,success_attackS);
% Wasps emerging that day:
```

```
emerged=horzcat(emerged_delay,success_attack0)...
    +horzcat(emerged_delay,success_attackS);

% Equations for state variables:
H0(t+1)=H0(t)+(bh-((bh-dh)*total_hosts(t)/k))*H0(t)-dh*H0(t)...
    -horizontal_acq(t)*H0(t)-success_attack0(t)...
    +(1-tv)*(1-cc)*(bh-((bh-dh)*total_hosts(t)/k))*HS(t)...
    +(1-tv)*(1-cc)*(1-ci)*(bh-((bh-dh)*total_hosts(t)/k))*HR(t);

HS(t+1)=HS(t)+tv*(1-cc)*(bh-((bh-dh)*total_hosts(t)/k))*HS(t)-dh*HS(t)
    ...
    +horizontal_acq(t)*H0(t)-fraction_attack(t)*HS(t)...
    +tv*(1-cc)*(1-ci)*(bh-((bh-dh)*total_hosts(t)/k))*HR(t);

HR(t+1)=HR(t)-dh*HR(t)-(1-pi)*(1-psp)*fraction_attack(t)*HR(t)...
    +(1-(1-pi)*(1-ps))*fraction_attack(t)*HS(t);

V0(t+1)=V0(t)+success_attack0(t)-killed0(t);

VS(t+1)=VS(t)+success_attackS(t)-killedS(t);

P(t+1)=P(t)+emerged(t)-dp*P(t);

end
```

B The MATLAB code for the implementation of the "all-or-nothing" encapsulation model

B.1 Basic encapsulation model

The basic MATLAB encapsulation model script is:

```
% Modelling Godfray and Hassell encapsulation with pea aphid parameters

%Time
DAYS=250;
t=(1:1:DAYS);
n=0.75; % change as needed
lambda=3; % value is approx 3
a=146;
b=0.0011;

% Preallocating for speed...state equations later in MATLAB script
% State variables:
N=zeros(size(t));
P=zeros(size(t));

% Initial populations
 N(1)=25;
 P(1)=10;

%Equations for state variables
for t=1:DAYS
```

```
% Type II functional response
N(t+1)=N(t)*lambda*(exp(-a*P(t)/(1+a*b*(N(t))))+n*(1-exp(-a*P(t)/(1+a*b
    *(N(t))))));
P(t+1)=N(t)*(1-n)*(1-exp(-a*P(t)));
end

% Plotting dynamics v time
figure(2)
plot(N,'red')
hold on
plot(P)
% Plotting phase plane
figure(3)
plot(N,P)
```

B.2 Basic evolution of encapsulation response model

The basic MATLAB evolution of encapsulation response model script is:

```
% Modelling Godfray and Hassell evolution model

%Time
DAYS=250;
t=(1:1:DAYS);
n1=0.3;
n2=0.3;
lambda1=4.5;
lambda2=4.5;
a=146;
K=1000;

% Preallocating for speed...state equations later in MATLAB script
% State variables:
N=zeros(size(t));
S=zeros(size(t));
P=zeros(size(t));

% Initial populations
 N(1)=25;
 S(1)=25;
```

```
 P(1)=10;

% Equations for state variables
for t=1:DAYS
N(t+1)=N(t)*lambda1*exp(-(N(t)+S(t))/K)*(exp(-a*P(t))+n1*(1-exp(-a*P(t)
    )));
S(t+1)=S(t)*lambda2*exp(-(N(t)+S(t))/K)*(exp(-a*P(t))+n2*(1-exp(-a*P(t)
    )));
P(t+1)=N(t)*(1-n1)*(1-exp(-a*P(t)))+S(t)*(1-n2)*(1-exp(-a*P(t)));

end

figure(2)
plot(N,'red')
hold on
plot(S,'green')
hold on
plot(P)
```

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